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IMPLEMENTATION OF METHODOLOGIES FOR REMOVAL OF VETERINARY PHARMACEUTICALS RESIDUES FROM WWTPs EFFLUENTS OF THE LIVESTOCK INDUSTRY



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RESUMO

A preocupação com os efeitos dos produtos farmacêuticos no ambiente tem vindo a aumentar largamente nos últimos anos. Uma vez que os fármacos são usados não só para o tratamento mas também para a prevenção de doenças, o consumo dos mesmos está em crescimento. Novas substâncias farmacologicamente ativas são desenvolvidas continuamente com destinos e efeitos no ambiente desconhecidos. Estes fatores levam a que os fármacos sejam considerados atualmente poluentes emergentes.

A utilização de fármacos veterinários tornou-se prática integrante da crescente indústria pecuária. Entre outros, os antibióticos são o grupo de compostos mais consumidos, sobre os quais existe uma crescente preocupação por parte da sociedade e comunidade científica devido ao aumento globalizado de bactérias resistentes a antibióticos. Os fármacos veterinários, embora utilizados numa extensão semelhante às dos de consumo humano, encontram-se muito menos estudados em termos de emissão e efeitos para o ambiente. Além disso, a maioria dos estudos existentes acerca de processos alternativos e/ou complementares às estações de tratamento de águas residuais (ETARs), tais como os processos avançados de oxidação ou os leitos de macrófitas, para reduzir a quantidade de fármacos libertados para o ambiente, têm sido dirigidos para as drogas de consumo humano.

Na base deste projeto de doutoramento estiveram dois fatores chave: (1) a necessidade de técnicas e metodologias analíticas capazes de detetar fármacos veterinários em diferentes matrizes ambientais; e (2) a necessidade de tecnologias adequadas para a remoção destes contaminantes emergentes dos efluentes das ETARs.

Para selecionar as drogas alvo de estudo, uma vez que não existe nenhuma listagem nacional com a priorização destas substâncias, utilizou-se uma listagem com os fármacos veterinários mais utilizados em Portugal, obtida a partir de criadores nacionais de suínos.

Os resultados obtidos neste trabalho indicaram que a cromatografia gasosa não será uma técnica adequada para a análise de um conjunto de fármacos veterinários inicialmente escolhidos, onde se incluíram enrofloxacin (ENR) e ceftiofur (CEF). Assim, posteriormente, foi otimizado e caracterizado um método analítico expedito para a análise simultânea de cinco fármacos de três famílias diferentes, nomeadamente, minociclina (MNC), oxitetraciclina (OTC), tetraciclina (TET) (todos da família das tetraciclinas), ENR (da família das fluoroquinolonas) e CEF (da família das cefalosporinas) em amostras aquosas, por extração em fase sólida (SPE) seguida de cromatografia líquida de alta eficiência (HPLC) acoplada a um detetor de díodos (DAD). Os limites de deteção e quantificação do método são suficientes para permitir a sinalização de níveis problemáticos das drogas em efluentes de águas residuais.

A metodologia SPE-HPLC-DAD foi aplicada a amostras de efluentes de diferentes ETARs com probabilidade de ocorrência de fármacos veterinários. Confirmou-se que os efluentes de algumas ETARs podem ser uma importante fonte de libertação destes compostos para o ambiente.

Uma metodologia analítica expedita foi também otimizada para a análise simultânea dos fármacos veterinários previamente selecionados (MNC, OTC, TET, ENR e CEF) em amostras sólidas ambientais, nomeadamente lamas de ETARs e sedimentos. A metodologia permite a análise dos referidos fármacos, com níveis aceitáveis de seletividade e de recuperação (tendo em conta a matriz complexa das amostras sólidas) sendo os respetivos valores da ordem de grandeza de outros métodos disponíveis na literatura aplicáveis a amostras similares. O método pode também ser utilizado para otimizar os processos envolvidos no tratamento de fármacos veterinários em reatores de lamas ativadas de ETARs ou leitos de macrófitas e ainda para quantificar os mesmos em sedimentos dos aquíferos que recebem os efluentes de ETARs.

Um estudo com reatores de lamas ativadas em sistemas de microcosmos mostrou que o principal fator a influenciar a eficiência da remoção destes poluentes da água é a sua capacidade de interação com as partículas sólidas. Os reatores com lamas, dopados com uma carga inicial de droga de $100 \mu\text{g L}^{-1}$, apresentaram percentagens de remoção de 68% para a ENR e 76% para a TET, após um período de 10 dias. Estes valores indicam que concentrações consideráveis, na ordem dos $\mu\text{g L}^{-1}$, persistem na fase aquosa após o tratamento nas ETARs e, consequentemente, os fármacos são libertados para o meio ambiente. Os potenciais riscos associados a efluentes contaminados com níveis vestigiais destes poluentes, nomeadamente antibióticos, são incertos, revelando a necessidade de tecnologias mais eficientes nas ETARs.

Os sistemas de leito de macrófitas foram estudados como passo de polimento direcionado à remoção de fármacos dos efluentes das ETARs. Inicialmente, testes simples em microcosmos apenas com a planta *Phragmites australis* evidenciaram que a mesma pode desempenhar um papel importante na remoção de fármacos do meio aquático. Posteriormente foram utilizados sistemas completos (planta e meio de suporte), em microcosmos, para estudar o potencial dos sistemas de leito de macrófitas para a redução/eliminação dos fármacos veterinários de águas residuais da indústria pecuária e de matadouros, água residual com uma matriz muito mais complexa que a existente nas ETARs urbanas. Percentagens de remoção de 94 e 98% foram registadas para TET e ENR, respetivamente. Estes valores foram consistentes ao longo de doze semanas de funcionamento a tratar água residual de uma suinicultura dopada com fármacos na ordem dos $100 \mu\text{g L}^{-1}$. Os resultados não mostraram qualquer indício de fitotoxicidade para a planta *P. australis* nos microcosmos. O mecanismo principal de remoção das drogas será a adsorção ao substrato. No entanto, para a TET existem também indícios da ocorrência de degradação. Assim, foi possível concluir que os sistemas de leito de macrófitas têm potencial para serem utilizados na mitigação da emissão de fármacos veterinários, nomeadamente ENR (uma fluoroquinolona) e TET (uma tetraciclina) de águas residuais.

Os resultados obtidos levam a concluir que os sistemas de macrófitas terão capacidade para reduzir os níveis de antibióticos de águas residuais da indústria agroalimentar. A avaliação de sistemas de leito de macrófitas em escala real, assim como dos processos pelos quais estes sistemas contribuem para a remoção dos fármacos veterinários, no sentido de serem potenciados/melhorados, merecem ser alvo de investigação no futuro.

ABSTRACT

The interest on the effects of pharmaceutical products on the environment has been largely increasing in the past years. Use of pharmaceuticals is growing as they are used not only for treatment but also for prevention of illnesses. New pharmacologically active substances are being developed constantly with unknown fates and effects on the environment. These reveal pharmaceuticals as continuous emerging pollutants.

The use of veterinary pharmaceuticals has become integral to the growing animal food industry. Among those, antibiotics are the most widely used, for which there is a growing social and scientific concern due to the worldwide increase in antibiotic resistant bacteria. Veterinary drugs are used in similar extent as human ones but, from an environmental point of view, drugs release rates and effects are less studied. In addition, studies on alternative and/or additive treatments to wastewater treatment plants (WWTPs), such as advanced oxidation processes or constructed wetlands (CWs), to reduce the input of drugs to the environment have been directed to human pharmaceuticals.

Two key factors were on the base of this PhD project: (1) the need for efficient analytical technologies and methodologies for veterinary drugs analysis in environmental samples; (2) the need for adequate technologies in WWTPs in order to sufficiently eliminate these emerging trace contaminants from effluents.

A list of veterinary drugs commonly applied in Portugal, obtained from national swine livestock producers, was used for drugs selection, since no Portuguese prioritization list for these substances exist at the moment.

A set of results obtained in this work suggested that gas chromatography (GC) is not a suitable technique for the analysis of a first group of veterinary drugs selected, which included enrofloxacin (ENR) and ceftiofur (CEF). So, afterwards, an expeditious analytical method for simultaneous analysis of five pharmaceuticals of three different families, namely minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), ENR and CEF, in aqueous samples through solid-phase extraction (SPE) followed by high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) was optimized and characterized. Detection and quantification limits obtained are sufficient for signaling problematic wastewaters effluents.

SPE-HPLC-DAD methodology was applied to samples of different WWTPs effluents with probability of containing veterinary drugs. The detection of some of the analyzed drugs in some samples showed that these effluents can be an important source of these drugs to the environment.

An expeditious analytical method for the simultaneous analysis of the previous selected five veterinary pharmaceuticals (MNC, OTC, TET, ENR and CEF) in environmental solid samples, including WWTPs sludge and sediments, was also optimized. Methodology yielded acceptable selectivity and recovery taking in account the complexity of solid matrices, in accordance with data available in the literature for similar analytical processes. These optimized methodologies

can be applied to studies on the fate of veterinary drugs in activated sludge processes of WWTPs, constructed wetlands beds and WWTPs recipient water bodies.

Activated sludge reactors microcosms systems study provided consistent results with the known fact that the major factor influencing the efficiency of pharmaceuticals removal from water is their ability to interact with solid particles. Sludge reactors with $100 \mu\text{g L}^{-1}$ initial drug doping charge presented removal rates of 68% for ENR and 76% for TET after a 10-days period. However, these values represent still considerable concentrations at $\mu\text{g L}^{-1}$ level that remain in the aqueous phase and consequently will pass through the WWTP to the receiving environment. The risks associated with effluents containing trace pollutants such as antibiotics remains uncertain, pointing out the need for more efficient WWTPs technologies.

Then, CWs were studied as polishing step to remove veterinary drugs from WWTPs effluents. Initial simplified microcosm tests with the plant *Phragmites australis* denoted that the plant could play an important role in drugs removal from the aquatic medium. Further tests were performed with complete CWs systems (plant and support system), at the microcosm scale, to study the potential positive effects of these systems for the reduction/elimination of veterinary pharmaceuticals from livestock and slaughterhouse industries wastewater, a wastewater with a matrix much more complex than that of urban wastewaters. Consistent removal efficiencies of 94 and 98 % were achieved for TET and ENR, respectively, treating pigfarm wastewater effluent doped at a $100 \mu\text{g L}^{-1}$ drug level, along twelve weeks. Results showed no occurrence of phytotoxicity in *P. australis* of CWs microcosms. Occurrence of adsorption of the drugs to the substrate may be the predominant mechanism for ENR removal, although for TET there were signs that degradation was also occurring. Therefore, results revealed that CWs have potential to mitigate the release of veterinary drugs, namely ENR (fluoroquinolone) and TET (tetracyclines) from wastewaters.

These last results point to a positive potential for CWs ability to remove veterinary antibiotics from wastewaters from livestock and slaughterhouse industry. Therefore, evaluation of full-scale CWs systems, as well as ways to enhance the processes by which CWs contribute for veterinary drugs removal deserve to be further investigated.

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ABBREVIATIONS AND SYMBOLS

AA	acetic anhydride
CEF	ceftiofur
COD	chemical oxygen demand
CW	constructed wetland
DAD	diode array detector
DMS	dimethyl sulfate
ECF	ethyl cloroformate
ENR	enrofloxacin
FLU	flufenamic acid
FID	flame ionization detector
FWS CWs	free water surface flow constructed wetlands
GC	gas chromatography
HPLC	high-performance liquid chromatography
HRT	hydraulic retention time
IBU	ibuprofen
IVE	ivermectin
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
OTC	oxytetracycline
MAE	microwave assisted extraction
MNC	minocycline
MS	mass spectrometry detector
MS ²	tandem mass spectrometry
PEN G	penicillin G
PEN V	penicillin V
RSD	relative standard deviation
SPE	solid-phase extraction
SPME	solid-phase microextraction
SSHF CWs	subsurface horizontal flow constructed wetlands
SSVF CWs	subsurface vertical flow constructed wetlands
TCC	total cell counts
TET	tetracycline
TSS	total suspended solids
USE	ultrasonic extraction
VA	vortex agitation
VSS	volatile suspended solids
WWTP	wastewater treatment plant

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PART I

INTRODUCTION

Chapter 1 - General Introduction

- 1.1 Background**
- 1.2 Veterinary pharmaceutical compounds**
- 1.3 Prioritizing veterinary medicines**
- 1.4 Ecotoxicology of pharmaceutical compounds**
- 1.5 Analytical methodologies**
- 1.6 Wastewater treatment plants (WWTPs)**
- 1.7 Constructed wetlands (CWs)**
- 1.8 Objectives**
- 1.9 Organization of the dissertation**
- References**

1.1 Background

The continued exponential growth in human population has created a corresponding increase in the demand for the Earth's limited supply of freshwater. Thus, protecting the integrity of water resources is one of the most essential environmental issues of the 21st century. Contamination of freshwater aquatic environments by chemicals remains a challenging issue both for professionals concerned about protecting our environment and also for members of the public, for whom freshwaters are a source of food, drinking water, and recreation (Kim and Aga 2007).

Recent decades have brought increasing concerns for potential adverse human and ecological health effects resulting from the production, use and disposal of numerous chemicals that offer improvements in industry, agriculture, medical treatment, and even common household conveniences (Kolpin et al. 2002). Due to their occurrence in trace amounts, the presence of pharmaceuticals in the environment as pollutants has been overlooked in the past years (Carlsson et al. 2006b). However, with the advent of sensitive state-of-the art analytical instrumentation, trace levels analysis of these compounds is increasing. Recent research investigations suggest that even low concentrations of drugs in the environment exert adverse ecological and human health effects (Kim and Aga 2007). In fact, concern on pollution by pharmaceuticals has grown after confirmation of their presence and ability to pseudo-persist in the environment (Fatta-Kassinos et al. 2011), namely in fresh water resources. Occurrence of medical substances in ground water, river water, sediments, soils and oceans was already reported two decades ago (Halling-Sørensen et al. 1998). More recently, Zuccato and co-workers showed that illicit drugs are also common contaminants of the aquatic environment of populated areas (Zuccato et al. 2008).

Pharmaceuticals, due to their possible toxic effects on the environment, can be divided into the following groups: antibiotics, hormones, analgesics and anti-inflammatory drugs, chemical compounds used for disinfection and cleaning, and endocrine-disrupting compounds (Kot-Wasik et al. 2007). Most active ingredients in pharmaceuticals are transformed only partially in the body and thus are excreted as a mixture of metabolites and bioactive forms into the sewage systems (Kim and Aga 2007). Household chemicals, pharmaceuticals, and other consumables as well as biogenic hormones are released directly to the environment after passing through wastewater treatment processes (via wastewater treatment plants, or domestic septic systems), which often are not designed to remove them from the effluent (Kim and Aga 2007, Kolpin et al. 2002).

The introduction of treated domestic wastewater into water resources has become common in urban areas, with several water recycling programs being operated. As reuse of treated wastewater increases, concerns that antibiotics and resistant bacteria are introduced into the drinking water systems have also escalated due to potential risk of human exposure (Halling-Sørensen et al. 1998). There are also new concerns that antibiotics will decrease

biodegradation of leaf and other plant materials, which serves as the primary food source for aquatic life in rivers and streams (Richardson 2012).

In water, bacteria from different origins (human, animal, environmental) are able to mix, and resistance evolves as a consequence of promiscuous exchange and shuffling of genes, genetic platforms, and genetic vectors. At the same time, antibiotics, disinfectants, and metals are released in water, and might exert selective activities, as well as ecological damage in water communities, resulting in antibiotic resistance (creation of “Super Bugs”) (Baquero et al. 2008).

Nevertheless, very few risk assessments of organic microcontaminants (present at trace, ng L⁻¹ levels), such as pharmaceuticals, have been carried out, or exist in the public domain (Daneshvar et al. 2012, Johnson et al. 2008, Kar and Roy 2011).

The main research on emerging pollutants on environment has been focused on human medications, personal care products and industrial endocrine disrupting chemicals while research on veterinary pharmaceuticals has been neglected.

1.2 Veterinary pharmaceutical compounds

The increasing demand for food and fiber has pushed agricultural industry toward using more and more organic and inorganic chemicals, namely application of veterinary pharmaceuticals in the growing animal food industry (Boxall et al. 2003). Veterinary medicines are used worldwide to protect animal health, prevent economic loss and help ensure a safe food supply (Boxall et al. 2003).

A variety of drugs and feed additives approved for use in food-animal agriculture fall into several pharmacological categories including antimicrobials, anti-inflammatories, parasiticides, anesthetics, sex hormones, antiseptics, bronchodilators and antifungals, and are delivered to the animals through feed or water, by injection, implant, drench, paste, orally or topically (Campagnolo et al. 2002, Díaz-Cruz and Barceló 2007, Sarmah et al. 2006). These categories include avermectins, quinolones/fluoroquinolones, antifolates drugs, aminoglycosides, tetracyclines, macrolides, streptogramins, amphenicol, β -lactams, among others.

Of the drugs approved for agriculture, antibiotics are among the most widely administered for animal health and management. The term ‘antibiotic’ is normally reserved for a diverse range of compounds, both natural and semi-synthetic, that possess antibacterial activity. In the European Union (EU) in the 1990s, a total use of 5 million kg of antibiotics has been reported, 3.5 million kg for therapeutic purposes and 1.5 million kg as feed or growth promotion additives (Zhao et al. 2010 and references therein). The worldwide increase in antibiotic resistant bacteria has led to social and scientific concern that the over prescription and misuse of human prescribed antibiotics and the increased and wide-spread use of sub-therapeutic doses of antibiotics in agriculture are responsible for this trend (Campagnolo et al.

2002, Sarmah et al. 2006).

As referred above, after usage, pharmaceuticals may reach waterways and possibly pose environmental problems. It is estimated that, for instance, 75% of antibiotics administered to animals are not absorbed and are excreted in waste. The wastes produced in animal farms may also be significant sources of endogenous hormones, as well as hormones administered to treat the animals and to promote growth. Given the strong link between steroid sex hormones (estrogens) and endocrine disruption in marine and freshwater fish, it is essential to assess their loads into the environment (Díaz-Cruz and Barceló 2007). Several studies have reported the occurrence of various veterinary pharmaceuticals in surface waters in Canada, The United States of America (USA), Europe and Asia (Hussain et al. 2012) as well as in groundwater, and in waste water treatment plant (WWTP) effluents (Kim et al. 2008). Garric et al. (2007) have found that veterinary drugs can be very potent to aquatic organisms as they observed effects on reproduction in the crustacean *Daphnia magna* at low levels as 1 pg L⁻¹ of the parasiticide ivermectin.

The pathway of veterinary pharmaceuticals to waterway is different from human pharmaceuticals. While human pharmaceuticals discharge into the environment mainly through sewage treatment plants, veterinary pharmaceuticals can enter the environment not only through livestock waste treatment plants but also through direct application in aquaculture, wash-off from topical treatments and via the application of sludge or animal manure (containing excreted products) to land (Boxall et al. 2003, Kim et al. 2008). In fact, the usage of treated sludge's and manure applications (biosolids) as fertilizers are considered significant sources of these pollutants to the environment (Speltini et al. 2011).

Once released into the environment, these pharmaceuticals and their metabolites may run into surface waters or leach to groundwater where they may affect the ecosystem as well as human health (Koschorreck et al. 2002).

1.3 Prioritizing veterinary medicines

The large number and wide variety of veterinary drugs available mean that it is difficult to identify those substances that are likely to have the greatest potential to impact the environment. The impact of a veterinary drug on the environment will be determined by a range of factors including the quantity used, the degree of metabolism in the animal, the degradation during storage of manure prior to land spreading and the toxicity of the substance to terrestrial and aquatic organisms (Boxall et al. 2003). Veterinary antibiotics are designed to affect mainly microorganisms and bacteria found in animals. Therefore, this property makes them potentially hazardous to other organisms found in the environment (Sarmah et al. 2006).

Several studies have been developed for identifying substances that may pose a risk

to terrestrial and aquatic systems, ie. their prioritization (Sanderson et al. 2004). The first approach by Boxall et al. (2003) has been applied in the UK using information on tonnage sold, typical usage regimes, metabolism and toxicity to aquatic and terrestrial organisms. Eleven substances, including antibiotics and ectoparasiticides, have been identified as high priority and 45 more substances have been identified as potentially high priority but further data was required (Boxall et al. 2003). Later, the same research group developed a scheme to prioritize veterinary drug active ingredients on the basis of their potential for indirect exposure to the general population via the environment and their toxicity profile (Capleton et al. 2006). Veterinary pharmaceuticals were prioritized also in Korea by their usage, potential to enter the environment and toxicological hazard (Table 1). Twenty compounds were identified in the top priority class, most of which antibiotics (Kim et al. 2008).

An estimated 6051 tons of active substances in veterinary drugs were used in 2004 in

Table 1 Veterinary pharmaceuticals assigned on top priority group in Korea and United Kingdom (adapted from (Kim et al. 2008)).

Korea	United Kingdom	
Ecosystem and Human health ^a (This study)	Ecosystem ^a (Boxall et al., 2005)	Human health ^a (Capleton et al., 2006)
Compounds		
Amoxicillin	Amoxicillin	Albendazole
Apramycin	Apramycin	Amoxicillin
Bromhexine	Bronopol	Baqueloprim
Ciprofloxacin	Doramectin	Chlorhexidine
Diclazuril	Enrofloxacin	Levamisole
Dihydrostreptomycin sulfate	Eprinomectin	Monensin
Doxycycline	Fenbendazole	Nitroxylin
Enramycin	Florfenicol	Procaine benzylpenicillin
Erythromycin	Ivermectin	Procaine penicillin
Fenbendazole	Lincomycin	Salinomycin sodium
Florfenicol	Monensin	Sulfadiazine
Fluvalinate	Moxidectin	Toltrazuril
Ivermectin	Oxytetracycline	Trimethoprim
Monensin sodium	Sulfadiazine	
Norfloracin	Tiamulin	
Oxytetracycline	Tilmicosin	
Spectinomycin	Trimethoprim	
Toltrazuril	Tylosin	
Tylosin		
Virginiamycin		

Compound names shown in bold indicate veterinary pharmaceuticals that appeared in common both in Korea and U.K.

^a Entity to protect.

the European Union. Kools and co-workers discussed available data sources and quality, as well as, their method and possible refinement of their approach for environmental risk-based ranking. Antibiotics and antiparasitic compounds were pointed as the two most used types of compounds. The lack of detail and the high uncertainty on the use of veterinary pharmaceuticals has made a careful environmental assessment difficult (Kools et al. 2008).

In Portugal this type of approach has not been attempted yet, as far as my knowledge. Contacts were performed with the responsible institution in Portugal for the veterinary drugs usage, *Direcção-Geral de Alimentação e Veterinária (Ministério da Agricultura, do Mar, do*

Ambiente e do Ordenamento do Território), but data on this matter, like the type of the more used drugs, or the more used drug, and the amounts annually consumed, were not available.

The company *CQO Plus - Engenharia, Ambiente e Energia Lda* arranged among Portuguese swine livestock producers a list of veterinary drugs commonly applied in Portugal. From the extensive list obtained, and by cross-referring with the available prioritization lists, research on this project was pointed on ivermectin (of the families of avermectins), which is an antiparasitic, and on the antibiotics enrofloxacin (a fluoroquinolone), neomycin and streptomycin (two aminoglycosides), tetracycline, oxytetracycline and minocycline (tetracyclines), penicillin G and penicillin V (penicillins) and ceftiofur (a cephalosporin, a class of β -lactams).

During the evolution of the PhD project, as result of analytical methodology achievements and further results obtained, the research focused essentially in enrofloxacin (ENR), tetracycline (TET) and ceftiofur (CEF) (Figure 1), as indicators of three different pharmaceutical families types, fluoroquinolones, tetracyclines and cephalosporins, respectively.

1.4 Ecotoxicology of pharmaceutical compounds

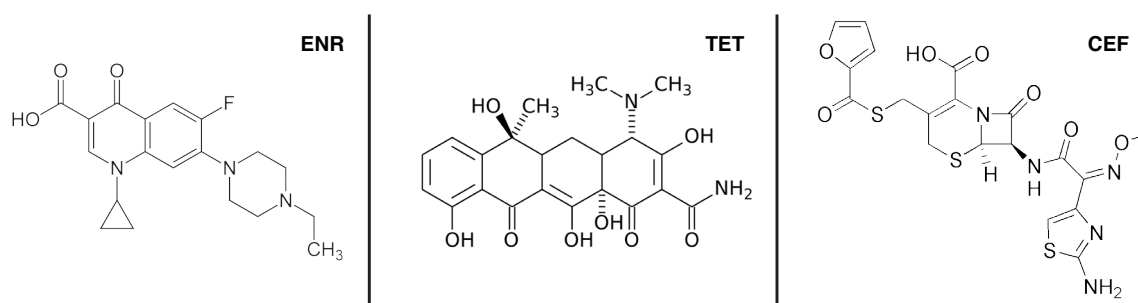


Figure 1 Enrofloxacin (ENR), tetracycline (TET) and ceftiofur (CEF) structural formulas.

Pharmaceuticals are designed to have a specific mode of action, and many of them for some persistence in the body. The modes of action of most pharmaceuticals in humans, animals and fish are often poorly understood; even more unknown is the possible effect on non-target receptor organisms (side effects). Moreover, the mixing of drugs might produce synergistic effects (Díaz-Cruz et al. 2003). These features, among others, make pharmaceuticals to be evaluated for potential effects on aquatic flora and fauna (Fent et al. 2006b). Data on chronic ecotoxicity are scarce and the scientific community understanding of potential ecotoxic effects mediated by pharmacological mechanisms of action is poor (Carlsson et al. 2006a). Discussion of the actual procedure on pharmaceuticals, especially on the need of appropriate ecotoxicity tests has been pointed as an important issue (Ferrari et al. 2004). Aquatic organisms are

particularly important targets, as they are exposed, via wastewater residues, their whole life. Standard acute ecotoxicity data have been reported for a number of pharmaceuticals, however, such data alone may not be suitable for specially addressing the question of environmental effects and, subsequently, to contribute for hazard and risk assessment (Fent et al. 2006b).

Hormonal activity of pharmaceuticals occurring in the aquatic environment has not yet been reported. Generally, the potential estrogenic activity of pharmaceuticals remains elusive. As these substances are present in the environment as mixtures and at very low concentrations, the analysis of mixture effects is of particular interest (Fent et al. 2006a).

Carlsson and co-workers pointed out that the potential environmental effects of an active pharmaceutical ingredients should not only be evaluated at the time of submission of the marketing authorization application but also be reviewed and re-assessed regularly during its lifecycle (Carlsson et al. 2006a). Tilghman et al. (2009) revealed the need for consistent pan-European screening programs designed for preliminary assessment and identification of the hazard from emerging pharmaceutical pollutants. Novel tools as ecotoxicogenomics and metabolomics, *in vitro* and *in vivo* bioassays and biomarkers in aquatic organisms together with bioinformatic and statistical clustering methods and toxicity identity evaluation (TIE)/ effect-directed analysis (EDA) approaches should be included in these programs.

The development of resistance to antimicrobial agents by many bacterial pathogens has compromised traditional therapeutic regimens, making treatment of infections more difficult. Three factors have contributed to the development and spread of resistance: mutation in common genes that extend their spectrum of resistance, transfer of resistance genes among diverse micro-organisms, and increases in selective pressures that enhance the development of resistant organisms (Halling-Sørensen et al. 1998). More than 90% of bacterial strains originated in seawater are resistant to more than one antibiotic, and 20% are resistant at least to five. The study of antibiotic resistance in indigenous water organisms is important, as it might indicate the extent of alteration of water ecosystems by human action. The introduction (and progressive accumulation) in the environment of antimicrobial agents, detergents, disinfectants, and residues from industrial pollution, as metals, contributes to the evolution and spread of such resistant organisms in the water environment (creation of “Super Bugs”) (Baquero et al. 2008). Recently, one antibiotic (erythromycin) has been included in the U.S. EPA’s final Contaminant Candidate List 3 as priority drinking water contaminant, on the basis of health effects and occurrence in environmental waters. In EU the revision of the priority substances list within the water framework directive (2000) describing the chemical status of European rivers, streams and lakes led to the inclusion of two pharmaceuticals (diclofenac and ibuprofen) (Richardson 2012).

Ecotoxicology of veterinary drugs is currently pointed as one underdeveloped field of research in which chemical analytical methodologies play a very important role.

1.5 Analytical methodologies

New methodological procedures and novel equipment in the field of analysis and environmental monitoring have made it possible in the past two decades to evaluate the level of environmental contamination, to identify the pollutants and to measure their concentration in different compartments (Debska et al. 2004). New analytical methods have better reproducibility and repeatability, and lower detection limits (Kot-Wasik et al. 2007). Residues of pharmaceuticals have most probably been present in our environment for quite a long time, but only recently instrumental analytical chemistry has made such progress that low ng L^{-1} concentrations can be reliably measured in various types of water samples (Buchberger 2007) or ng Kg^{-1} in other environmental matrixes (Richardson 2012). Richardson has been publishing since 2000 under the thematic of emerging contaminants water analysis and has observed that the attention on pharmaceuticals analysis is clearly increasing through the past 12 years (Richardson 2000, 2002, 2003, 2004, 2006, 2007, 2008, 2009, 2010, 2012, Richardson and Ternes 2005, 2011).

Analysis of pharmaceuticals poses a difficult challenge to the researchers for the following reasons: the great variety of pharmaceutical compounds; the need to identify not only the pollutants in question but also their derivatives and metabolites; the diversity of matrices (e.g., sediment, sludge, surface water, wastewater and biological samples) and differences in pollution-load levels; the possibility of interference between sample components of similar physicochemical characteristics that are present in the same sample at different concentrations; and, the lack of suitable standards and certified reference materials (Kot-Wasik et al. 2007).

When residue analysis of pharmaceuticals became an important issue in the 1990s, gas chromatography (GC) was the preferred chromatographic technique together with various derivatization procedures for the analytes. Although GC with mass spectrometry (MS) may still be the perfect technique for certain classes of pharmaceuticals, high-performance liquid chromatography (HPLC) and liquid chromatography (LC) become the most applied techniques after 2007 (Buchberger 2007).

The application of advanced measurement technologies (e.g., GC-MS and GC with tandem MS (GC-MS²) or LC with MS (LC-MS) and LC with tandem MS (LC-MS²)) to environmental analysis has allowed the determination of a broader range of compounds, including pharmaceuticals, and has therefore permitted a more comprehensive assessment of environmental contaminants. The LC-MS² method offers an improvement over GC-MS(or MS²) since the derivatization step is avoided and limits of detection (LODs) lower than 1 ng L^{-1} can still be achieved. In general, LODs achieved with the LC-MS² methods are slightly higher than those obtained with the GC-MS. When an efficient derivatization is carried out, GC methods, especially GC-MS applications, are still powerful tools for the determination of several groups of pharmaceuticals in the ng or μg per litter levels. In addition, further innovations have also been made in rapid online extraction and bag extraction, as well as online derivatization techniques in combination with GC-MS(or MS²) detection (Richardson 2012). LC-MS², on the other hand, shows advantages

in terms of versatility and less complicated sample preparation. LC-MS² is becoming more commonly used in pharmaceuticals analysis because of its high sensitivity and its ability to confirm compounds. MS² detection is therefore preferred for increased analytical sensitivity and selectivity in complex matrices, such as wastewaters (Fatta et al. 2007). Nowadays, LC-MS² is the method of choice for the determination of all classes of pharmaceuticals in aqueous matrixes. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most commonly used LC interfaces. Major innovations have been made in modern hybrid MS systems (e.g., Fourier transform ion cyclotron resonance MS (FT-MS), quadrupole time-of-flight MS (Q-TOF-MS)) coupled to LC, providing accurate masses of the analytes and information for mass fragments, which can be used to identify the chemical structures.

Despite the fact that LC-MS is the preferable technique for achieving low levels of detection of pharmaceutical drugs, HPLC with different detectors, which is a primary tool of analysis in most service, commercial and research laboratories, has also been commonly applied (Malintan and Mohd 2006). This accessible technique (although less sensitive and selective than LC-MS and LC-MS²) has the potential to be applied for signaling problematic wastewaters effluents.

In fact, the analysis of the broad therapeutic classes of drugs consumed worldwide has involved the use of a wide variety of techniques. Several methods have been recently published, as well as good amount of reviews under this method thematic. For example, Díaz-Cruz et al. (2003) reviewed the methods used for analysis of pharmaceuticals in soils, sediments and sludge, namely through the use of GC, HPLC and LC. Buchberger (2007) discussed the novel analytical procedures for screening of drug residues in water, waste water, sediment and sludge, through the main chromatographic methods coupled to MS detection. Díaz-Cruz and Barceló (2007) focused a paper on the advances in LC-MS residue analysis of veterinary drugs in the terrestrial environment. Fatta et al. (2007) reviewed the developments and applications within water and wastewater environmental matrices analysis. Hao et al. (2007) reviewed GC-MS and LC-MS -based analytical methods used for the determination of bioactive pharmaceuticals and personal-care products in the environment. Kosjek et al. (2007) discussed capabilities, potentialities and limitations of different GC and LC mass analyzers. Richardson (2012) provided a biennial review covering the developments in environmental analysis using MS for emerging contaminants, including pharmaceuticals, over the period 2010–2011.

Besides chromatographic separation, further detection and respective data analysis, the analytical procedure usually comprises three previous steps: sampling, sample preparation for the extraction of the compound and separation of the compound through clean up of the extract (Pavlović et al. 2007).

The sample-preparation procedure is one of the most important parts of the analysis of organic compounds in environmental matrices. The first step in liquid sample preparation is filtration of an appropriate volume (typically between 50 and 1 L) through <1 µm glass-fiber filters to avoid extraction inefficiencies due to the presence of suspended solids. Extraction of

pharmaceuticals from the sample into a small volume of solvent is the next step (Fatta-Kassinos et al. 2011). Currently, solid-phase extraction (SPE) is the most popular, well-established sample-preparation technique, with which a good sensitivity is obtained. Alternative techniques to SPE (e.g., solid-phase microextraction (SPME) and liquid-phase microextraction (LPME)) have been applied due to several advantages that they have over SPE in terms of speed, ease of sample handling and minimizing solvent use. However, the precisions obtained have been lower, indicating the need for these techniques to be further optimized (Fatta et al. 2007). In a general overview SPE is adopted for every type of matrix pre-concentration and purification method.

In the case of solid environmental matrices, additional sample handling is required. The low environmental concentrations (usually ng g^{-1}) and the complexity of the solid matrices make heavy demands on the analytical work, especially in extraction, pre-concentration and purification processes (Díaz-Cruz and Barceló 2007). Especially, the monitoring of drugs in solid environmental samples normally requires the use of time-consuming and labor-consuming methodologies. The quality assurance of each step involved in the whole analytical procedure, including sampling and storage, is essential for the reliability of the analytical determinations that follow (Díaz-Cruz et al. 2003). The sampling of solid samples mainly sediment and sludge results in a solid as well as a liquid phase, so that separation by filtration or centrifugation may be necessary. Subsequently, the solid sample may undergo air-drying, drying by heating, or freeze-drying followed by grinding and sieving (Buchberger 2011).

Extraction of pharmaceuticals from solid samples has been done by conventional Soxhlet extraction, vortex agitation (VA), microwave assisted extraction (MAE), ultrasonic extraction (USE), or pressurized liquid extraction (PLE) (Tadeo et al. 2012). The isolation of analytes from environmental solid samples therefore encompasses optimization of the extracting conditions, especially temperature and pressure, in order to enhance solvents capacity to extract analytes from a variety of solid matrices. This extraction is done by dropping the surface tension and increasing analyte solubility and diffusion (Díaz-Cruz and Barceló 2007).

The USE is attractive because the equipment necessary is widely available and the extraction can be done with a reasonably small volume of solvent (typically 0.1–2 g sample treated with 5–25 mL of solvent) with an extraction time between 10 and 60 min. (Buchberger 2011). Usually, the process is sequentially repeated two or three times to achieve higher extraction efficiency, being the extracts afterwards combined for analysis. USE has the benefit of shortened extraction times compared to classical liquid extraction methods, however poor reproducibility due to lack of uniformity in the distribution of ultrasound energy has been reported (Pavlović et al. 2007).

The VA is used extensively as the solely extraction procedure (Hamscher et al. 2002, Ok et al. 2011) and also as sample homogenization step in varied procedures followed by USE or MAE (Hu et al. 2010).

The MAE involves heating solid sample-solvent mixtures in a closed vessel with

microwave energy under temperature-controlled and pressure-controlled conditions. This closed extraction system enables analyte extraction with elevated temperatures and pressures, accelerating the extraction process, yielding a performance comparable to the standard Soxhlet method (Pavlović et al. 2007). Although MAE introduces some complexity into the procedure, it allows the acceleration of the sample preparation step, provides higher recoveries and depends less on the nature of the sample (Andreu et al. 2007). The main parameters influencing MAE performance include the nature of the solvent and the matrix, solvent volume, microwave power, exposure time, and temperature (Sanchez-Prado et al. 2010).

The solutions obtained after sample extraction can contain co-extracted components and further purification is generally needed, to eliminate chromatographic interferences, as well as to improve method detection limits. Usually extracts from solid samples are purified according to the techniques employed for aqueous samples, which are mainly based on SPE (Díaz-Cruz and Barceló 2007, Pavlović et al. 2007).

Differentiation in methodologies for human or veterinary drugs is not a common procedure both for aqueous or solid samples. Although the initial advances in this field were directed for human drugs of common use, later the range of pharmaceuticals started to increase and to incorporate sometimes drugs of veterinary application. Nevertheless, nowadays, only a few studies are addressed solely for veterinary drugs. Matrices where veterinary drugs are commonly found, from an analytical point of view, can be more challenging than those for human drugs, namely due to the usual higher organic loads associated with animals residues.

1.6 Wastewater treatment plants (WWTPs)

Pharmaceuticals in their native form or as metabolites are continually being introduced into sewage waters, mainly indirectly in excreta or through disposal of un-used or expired drugs, or directly in discharges from pharmaceutical-manufacturing plants (Barceló 2007). The introduction of treated domestic wastewater into water resources, increases concern that antibiotics and resistant bacteria are introduced into the drinking water systems (Halling-Sørensen et al. 1998). At present, urban wastewaters are considered the most important source of pharmaceutical compounds in the aquatic environment.

The presence of several pharmaceuticals in WWTPs effluents has been confirmed in Germany, Netherlands, Switzerland, United Kingdom, France, Greece, Sweden, Italy, Spain, USA, Canada, Brazil and Australia (Castiglioni et al. 2006). WWTPs were designed to remove organic pollutants, mainly estimated as dissolved organic matter, solids and nutrients but not pharmaceutical compounds. For the majority of drugs, removal by conventional biological treatments seems inefficient, being these drugs found in significant amounts in WWTPs effluents and surface water receiving these effluents. The exposure of aquatic organisms to

pharmaceuticals and their metabolites may be significant in view of the often considerable levels occurring in treated effluents of WWTPs, contaminated waters and landfill leachates. In fact, widely used pharmaceuticals can be found in the ng L^{-1} to $\mu\text{g L}^{-1}$ range in municipal wastewaters (Carballa et al. 2004). A chronic exposure of wildlife at discharge sites to pharmaceutical residues can be expected (Fent et al. 2006a).

During municipal wastewater treatment, the waste stream is separated into two components, solids and liquid effluent. A large fraction of the total anthropogenic waste, including numerous organic contaminants such as pharmaceuticals, entering WWTPs ultimately may reside in the biosolids. Many of these organic contaminants have moderate to large octanol–water partitioning coefficients and therefore it can be predicted that they undergo hydrophobic partitioning into the organic-rich solids phase during wastewater treatment (Kinney et al. 2008). This can represent a significant environmental problem during utilization of biosolids. For instance, Kinney et al. (2008) demonstrated that organic contaminants, namely pharmaceutical compounds, could be transferred from source materials, such as biosolids, to soil-dwelling earthworms.

Nevertheless, despite sorption to sludge, a significant amount of the pharmaceuticals can still remain in the aqueous phase passing through the WWTPs. For instance, a study carried out in Italy, to assess the efficiency and identify factors affecting the removal of pharmaceuticals in WWTPs, revealed that the total amount of twenty-six drugs belonging to various therapeutic classes, and two metabolites, discharged into the environment ranged between 60 and 180 kg day^{-1} (Castiglioni et al. 2006). Another study indicated that the removal efficiencies of a WWTP located in the south of Spain surveyed for a group of 14 compounds varied from 20% (carbamazepine) to 99% (acetaminophen), but in all cases resulted insufficient to avoid their presence in treated water and, subsequently, in the environment (Gómez et al. 2007).

For veterinary drugs, inputs from manure application to soils and aquaculture activities are considered significant sources (Boxall et al. 2004). However, direct discharges of livestock industries effluents into the aquatic environment (directly drain out to rivers and lakes) can also be a significant source of these compounds in the environment (Tong et al. 2009). Slaughterhouses also produce wastewater-containing pharmaceuticals that can be discharged directly into a nearby river, after simple disposal, which may greatly influence the local environment (Shao et al. 2009). Several livestock industries and slaughterhouses have now WWTPs to deal with their effluents to treat organic, nitrogen and phosphorus loads. Similar to municipal WWTPs, most of these WWTPs are also not designed to remove pharmaceutical compounds. Although some of these effluents are directed to municipal WWTPs, most of them are directly discharged into the environment. Therefore, these WWTPs effluents must also be considered as a possible source of veterinary drugs and should be taken in consideration. Nevertheless, only a few recent works were devoted to the monitoring of these drugs in these types of effluents, mostly in Asian countries (Ben et al. 2008, Shao et al. 2009, Tagiri-Endo et al. 2009, Tong et al. 2009).

One important aspect to solve the load of pharmaceutical residues in wastewater and surface water is to optimize wastewater treatment processes. Since many pharmaceuticals end up in WWTPs, recent trends in the choice of proper wastewater treatment, evaluation of the efficiency of WWTPs, monitoring treated wastewaters, and finally, monitoring waters to which wastewater is discharged are of the greatest importance (Kot-Wasik et al. 2007). For example, several studies identify clofibric acid as a refractory contaminant of municipal WWTP (Dordio et al. 2009b). Clofibric acid is non-biodegradable, highly mobile and very persistent in the environment, with a half-life of 21 years and a water residence time of 1–2 years. Therefore, there is a growing need for alternative wastewater treatment processes for removing pharmaceuticals from waters. Advanced water reclamation systems (e.g. ozonation, photo-fenton and reverse osmosis) able to efficiently eliminate these pollutants have been developed (Sirés and Brillas 2012). However, these systems require a high level of energy consumption and are expensive to build and maintain. Constructed wetlands (CWs), natural wetland or polishing ponds systems can be managed as water quality improving systems as alternative or additive low-cost wastewater treatments (Dordio et al. 2010, Matamoros and Salvadó 2012, Meers et al. 2005).

1.7 Constructed wetlands (CWs)

“A wetland is an area where water is above, at, or just below, the ground’s surface during part, or all, of the year, so producing water-logged soil conditions.” (Price and Probert 1997). There are two types of wetlands, the natural occurring ones and those that have been purposely constructed. Historically, wetlands have been used (often unintentionally) to clean liquid effluent and also to provide mode of conveyance for water-based transportation, sewer for wastes, supply of nutrients to agricultural land (via flooding), coastal defenses and a buffer against flooding, habitat for wildlife, recreational areas and as aquaculture resource, through the introduction of fish and/or water-tolerant food crops.

Although the Ancient Egyptians and Chinese used naturally occurring wetlands to clean liquid effluent, the first record of a constructed wetland (CWs), which was used to treat drainage water from suburban houses, was in an essay written to the Head of the Hornsbury Literature Institute, New South Wales, Australia in 1904 (Price and Probert 1997). The scientifically based use of wetlands for wastewater treatment in CWs began in the 1950s and 1960s with investigations by K. Seidel and R. Kickuth in Germany ((Mander and Mitsch 2009 and references therein).

Constructed wetlands (Figure 2) are defined as engineered wetlands that utilize natural processes involving wetland vegetation, soil, and their associated microbial assemblages to assist, at least partially, in treating wastewater or other polluted water sources (Helt et al. 2012). These wetlands can be used for primary, secondary and tertiary treatment of municipal

or domestic wastewaters, stormwater, agricultural and industrial wastewaters (such as landfill leachate, petrochemicals, pulp and paper, food wastes and mining) usually combined with an adequate pre-treatment. Although they are widely used for municipal wastewater, the application to industrial wastewater has to be carefully analyzed since its composition is frequently highly variable and the treatment needs are not the same. However, the use of CWs for the treatment of industrial wastewaters has increased over the past ten years (Calheiros et al. 2007). In general, CWs are not used as the single treatment process in sewage treatment plants but are commonly used as part of the tertiary treatment step, after biological and chemical treatment (Breitholtz et al. 2012). The number of CWs receiving wastewater from municipal, industrial, agricultural, and stormwater sources has increased to more than 20,000 across the world until 2008.



Figure 2 Constructed wetland (CW) example for domestic wastewater treatment in rural areas.

When properly planned, these treatment wetlands offer opportunities to regain some of the natural functions of wetlands and offset some of the significant losses in the wetland area (Mander and Mitsch 2009). In fact, the main disadvantage of CWs is the large surface area per inhabitant needed, but the low operational and maintenance costs and easy exploitation make this technology very attractive (Matamoros and Salvadó 2012). But besides water quality improvement and energy savings, CWs have other features related to the environmental protection such as promoting biodiversity, providing habitat for wetland organisms and wildlife (e.g. birds and reptiles in large systems), and serving climatic (e.g. less CO₂ production) and hydrological functions and biomethylation (Schröder et al. 2007).

Performance efficiencies of constructed or natural wetlands depend on several variables, such as the quality and quantity of effluent to be treated and of the biological (biological degradation, plants and aquatic organisms' uptake), chemical (chemisorption, photo-decomposition and degradation) and physical (volatilization and sorption) processes

in that particular wetland system (Adhikari et al. 2011, Dordio et al. 2007, Meers et al. 2005). Depending on the type of CWs used, its design, water depth, the availability of electron acceptors, UV radiation, organic loading rate and hydraulic retention time (HRT) will also affect its removal capability (Breitholtz et al. 2012, Matamoros et al. 2008a). A concerted action among plants, microorganisms and matrix components can decrease the concentration of the compounds to levels that are safe for the aquatic biota or production of drinking water. The treatment efficiency of these systems is highly dependent on temperature, since microbial activity increases with increasing temperature (e.g. (Dordio et al. 2010)). Nevertheless, despite the changes that can occur throughout the year CWs generally meet relevant effluent discharge criteria even in colder climates (Healy et al. 2007).

The CWs are designed to take advantage of many processes that occur in natural wetlands, but with a more controlled approach offering relatively low investment and operation costs, while producing high quality effluent with less dissipation of energy (Helt et al. 2012). There are two types of CWs: free water surface (FWS CWs) and sub-surface flow (SSF) CWs. In FWS CWs, wastewater flows in a shallow water layer over a soil substrate. Sub-surface CWs can be either sub-surface horizontal flow CWs (SSHF CWs) or sub-surface vertical flow CWs (SSVF CWs) (Figure 3). In SSHF CWs, wastewater flows horizontally through the substrate while in SSVF CWs wastewater is dosed intermittently onto the surface of the substrate (normally sand and gravel layers that act like filters) and gradually drains through it, the water being collected in a drain at the base. CWs can be planted with a mixture of submerged, emerged and, in the case of FWS CWs, floating vegetation (Healy et al. 2007).

The potential of SSF CWs for the removal of contaminants occurring in urban wastewater has attracted increasing interest with a view of treating wastewaters from small populations to comply with environmental regulations such as the European Union Directive 91/271 and the U.S. EPA Clean Water Act and to attenuate diffuse agricultural contamination runoff to surface waters (Matamoros et al. 2005). Until recently, nitrogen and phosphorus were the primary constituents to be removed from effluents in wetland systems, with their concentrations depending on the source of wastewater and the extent of nonpoint source pollution (Oulton et al. 2010). The removal rate of microorganisms in CWs has shown varying degrees of effectiveness; however, several studies also illustrate the improvement of microbial water quality using CWs (Helt et al. 2012). Recently, findings in different studies on the treatment performance of different CWs such as HSSF, VSSF, FWS and hybrid systems, have demonstrated that CWs have a good capacity for removing a variety of micro-pollutants, including pharmaceuticals (Haarstad et al. 2012, Hijosa-Valsero et al. 2011a). CWs ability for pharmaceuticals removal, besides the work developed by Breitholtz and co-workers (Breitholtz et al. 2012) that accessed 92 pharmaceuticals on four full-scale FWS CWs in Sweden, has been accessed mainly for a small range of human pharmaceuticals by two groups, one in Spain (Environmental Chemistry Department - Institute of Environmental Assessment and Water Research (IDAEA)) and another in Portugal (Évora Chemistry Centre – Universidade de Évora). Portuguese group studied at

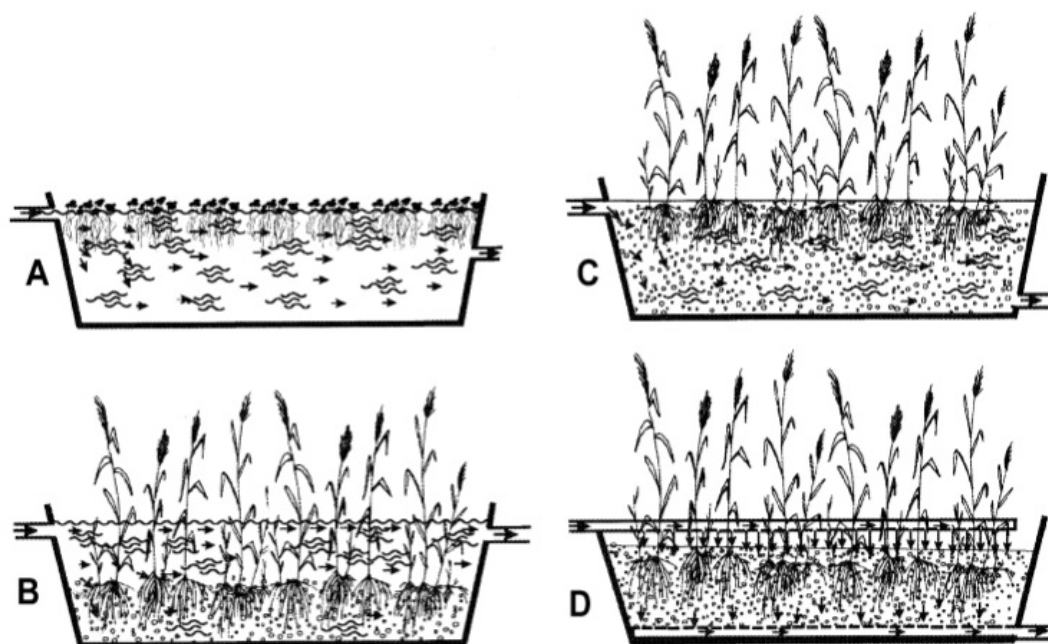


Figure 3 Wetland systems for wastewater treatment. Free water surface constructed wetlands (FWS CWs) A) with free-floating plants or B) horizontal surface flow wetland with emergent water plants; and sub-surface flow constructed wetlands (SSF CWs) C) sub-surface horizontal flow (SSHf CWs) D) sub-surface vertical flow (SSVF CWs) (adapted from (Stottmeister et al. 2003)).

microcosm level the removal of ibuprofen, carbamazepine, clofibric acid and atenolol (Dordio et al. 2010, Dordio et al. 2009a). The Spanish group has been evaluating mainly the removal of ketoprofen, naproxen, ibuprofen, diclofenac, salicylic acid, carbamazepine, clofibric acid, furosemide and caffeine at both full-scale (Hijosa-Valsero et al. 2010, Matamoros et al. 2009, Matamoros and Salvadó 2012) and mesocosm level (Hijosa-Valsero et al. 2011a, Matamoros et al. 2005, Reyes-Contreras et al. 2012). There are also a few more studies that addressed the removal of the compounds mentioned above in full-scale systems at USA (Conkle et al. 2008), Republic of Korea (Park et al. 2009) and Singapore (Zhang et al. 2011) and in mesocosms at UK (Reif et al. 2011). Regarding veterinary drugs, there is a scarcity of studies with only two recent papers devoted to this type of compounds. One of the studies focused on the removal of the veterinary antibiotics sulfonamides from swine wastewater by mesocosm constructed macrophyte floating bed systems (FWS CWs) (Xian et al. 2010). Another studied ionophores (pharmaceuticals used exclusively for veterinary application considered as high-risk compounds) removal by a mesocosm scale FWS CWs (Hussain et al. 2012). In addition, a study on surface flow CWs evaluation for 12 pollutants removal (including pharmaceuticals, personal care products and herbicides), from a WWTP plant secondary effluent provided also data on the veterinary drug flunixin (Matamoros et al. 2008b).

Depuration in CWs is achieved by the concerted action between plant roots and rhizomes, microorganisms and the solid media (substrate) components. These treatment systems provide different micro-environments where various removal processes can take place: physical (retention, adsorption on the substrate, adsorption on the biofilm, photodegradation, volatilization), chemical (degradation), vegetal (uptake, phytovolatilization, release of exudates, oxygen pumping to the rhizosphere, providing an adequate surface for biofilm growth) or microbiological (metabolization).

Traditionally, recommend substrates for FWS CWs is a substrate rich in iron, calcium and aluminum, for SSHF CWs is a soil or gravel, and in SSVF CWs is an active sand layer with a depth of ca. 1 m (Healy et al. 2007). Most studies on CWs use only mineral substrates such as sand and/or gravel as growing medium. However, these systems may be confronted with several problems, such as difficulties of plant settlement, low adsorption capacity and clogging. Therefore, other types of substrate are being tested such as slag and charcoal, or mixed media of mineral and organic substrates such as peat or only organic substrate (Wang et al. 2010). More recently, some choices of agro-industrial wastes (e.g. rice husk, pine bark) for the solid matrix of SSF CWs are being considered as interesting alternatives to other more common choices (e.g. gravel) due to the usually low-cost of such materials and the economical value for the local economies in finding reuses for such wastes. In addition, biosorbents (sorbents of natural origin, such as cork or peat) have been gaining in importance, as some of these materials can have an increased affinity for some types of contaminants and they are, in general, easily disposed of by incineration (Dordio et al. 2011b). The use of processed natural materials in CWs systems can present additional functions beyond the simple process of filtration. Their surface areas can constitute a support for microbial population growth in biofilters as well as for the development of the plants in the CWs. The efficiency of these biological systems for the removal of pollutants can, in fact, be significantly enhanced by a greater capability of the filter media to retain these compounds by sorption, ionic exchange or other physico-chemical processes. For example, previous studies have shown that light expanded clay aggregates (LECA) were able to remove human pharmaceuticals, clofibric acid, ibuprofen, carbamazepine, from water and wastewater by sorption (Dordio et al. 2009a, Dordio et al. 2009c, Dordio et al. 2007). Consequently, the selection of a medium with a high sorption capacity can be an important step in the optimization of the CWS performances. These capabilities will be dependent upon the chemical and physical properties of the material chosen (Dordio et al. 2007). However, the sorption of any compound to a fixed amount of solid matrix is not infinite. Thus, it is vital to understand the sorption of such compounds to the substrate within a treatment wetland over the long term (Conkle et al. 2010).

The choice of plants is also an important issue in CWs once they mediate important processes. For example, plant metabolic activity releases oxygen into the rhizosphere, which aids in nitrification through the direct uptake of nutrients. The access and availability of nutrients affects plant growth response and resource allocation, which influence removal

efficiency in wetlands. For example, nutrient removal can be optimized by selecting suitable species with higher capacities for absorption of inorganic nitrogen and phosphorus and conversion into plant biomass (Adhikari et al. 2011). However, plants must also survive the potential toxic effects of the wastewater and its variability (Calheiros et al. 2007). In general, mixed stands of plants will be more stress resistant than monocultures, establish a higher diversity of rhizobacteria and possibly increase the CWs efficiency (Schröder et al. 2007). Operation of flooded horizontal beds with floating species (*Eichhornia*, *Pistia*, *Lemna*), or the inclusion of halophytes or terrestrial plant species with different rooting depths in vertical flow beds has been also pointed out as a solution to improve CWs extraction efficiencies (Schröder et al. 2007).

The most widely used plants in CWs in North America are cattails (*Typha spp.*), bulrushes (*Schoenoplectus spp.*) and reeds (such as *Phragmites australis*) (Adhikari et al. 2011). At Europe the most common is the reed *P. australis* although other plant species, such as cattails bulrushes (*Scirpus spp.*) and reed canarygrass (*Phalaris arundinacea*) have also been used for both domestic and industrial wastewater treatment (Price and Probert 1997). In Portugal, the main macrophyte species used in CWs are *P. australis*, *Iris pseudacorus* (yellow iris) and *Cyperus spp.*. In some systems, *Juncus effuses* (soft rush), other *Juncus spp.*, and *Scirpus spp.* are also found to establish spontaneously (Calheiros et al. 2007).

Constructed wetlands can exploit the ability of plants to adsorb, uptake and concentrate inorganic elements and organic xenobiotics, as well as, to release root exudates that enhance organic compounds biotransformation and microbial degradation. For instance, several authors that have studied the importance of vegetation in removing metals from natural and constructed wetlands during wastewater treatment have shown that bioaccumulation processes are effective in reducing some metals such as arsenic and selenium (Adhikari et al. 2011). In addition, several studies have shown not only the capacity of plants, namely macrophytes ones, to withstand fairly high concentrations of organic xenobiotics but also their ability to remove them from contaminated waters (Dordio et al. 2009b). Due to these characteristics macrophytes are frequently chosen for the application of phytoremediation technologies to treat waters contaminated with organic pollutants. In fact, previous studies have shown that *Typha spp.*, presents a high capacity to tolerate and remove some pharmaceuticals from contaminated waters (Dordio et al. 2009b, Park et al. 2009), as well as *P. australis* (Ávila et al. 2010, Matamoros and Bayona 2006). Besides macrophytes, duckweeds have also been studied regarding pharmaceuticals removal in CWs. It was observed that these plants contributed to these pollutants removal by active plant uptake (both directly and indirectly affecting the fate of pollutants), by favoring microbial transformation and through passive plant-associated processes, specifically sorption (Reinhold et al. 2010). Whenever plants take up these contaminants, their location within the plant and the metabolic transformations and final form in which they are present in the tissues are still largely unknown. However, these issues are of practical relevance when considering the implementation of this technology, for example

when considering the need for harvesting plants to avoid the reentry of these pollutants in the environment through dead plant material (Dordio et al. 2011a).

As mentioned, macrophytes have shown an overall good tolerance to the exposure to contaminants and are capable of contributing to the removal of many of these substances (Hijosa-Valsero et al. 2011b), including pharmaceuticals. Until now, however, phytotechnologies have been only scarcely applied for pharmaceuticals depuration with only a few recent studies on the subject (Dordio et al. 2009a, Haarstad et al. 2012, Hijosa-Valsero et al. 2010, Matamoros and Bayona 2006, Park et al. 2009) and available data on phytotoxicity of pharmaceutical substances and their fate in plants is currently very limited (Boxall et al. 2006, Dordio et al. 2009b, Huber et al. 2009, Kong et al. 2007).

The generally high microbial biomass and activity in some wetland soils may also promote degradation of pharmaceuticals (White et al. 2006). The coexistence of several micro-environments in CWs allows for a variety of microbiological communities, which might be able to offer different metabolic pathways leading to pharmaceuticals degradation, which, however are still unclear (Hijosa-Valsero et al. 2011a).

Potential for photodegradation of the pharmaceuticals in FWS CWs also exists (Ryan et al. 2011). Several studies have documented photodegradation of a number of drugs in surface waters and, while light can be attenuated in lakes and rivers as it passes down through the water column, treatment wetlands are generally shallow systems that reduce attenuation of light and maximize photolysis (White et al. 2006).

In spite of the carried out works, the information on CWs efficiency in removing pharmaceuticals from wastewaters is limited to only a few compounds and plant species. Although growing, more research is needed to look more intensively at the role of substrates (including soil organic matter), microbial activity, plant uptake and biofilms on the sorption/degradation/removal of pharmaceutically active compounds from wastewater in wetland settings. Little attention has been paid to which kind of primary treatment is more efficient for pharmaceuticals removal from wastewaters. Moreover, several operational parameters and design characteristics of CWs, such as effluent feeding regime and the influence of climate, have not been optimized for pharmaceuticals removal yet. For instance, one of the few factors already known is that seasonality, namely summer conditions (e.g. warmth, plant activity and sunlight) enhance the removal of some pharmaceuticals (Hijosa-Valsero et al. 2011a). However, knowledge about temporal evolution of pharmaceuticals removal in CWs is also scarce (Reyes-Contreras et al. 2012). Besides, it is still unclear which specific mechanisms are responsible for pharmaceuticals elimination in CWs.

In addition, most available studies regarding pharmaceuticals removal in CWs are addressed to human drugs in municipal effluents. And, although a few FWS CWs studies are focused on pesticide removal from agricultural or urban runoff, few studies exist on veterinary drugs attenuation (Hussain et al. 2012, Matamoros et al. 2008b, Xian et al. 2010), particularly on removal of these drugs from livestock industry effluents. Therefore, more research on the

potentialities of CWs for removal of veterinary pharmaceuticals is in need.

1.8 Objectives

Use of pharmaceuticals is increasing, as they are used not only for treatment but also for prevention of illnesses. New pharmacologically active substances are being developed constantly with unknown fates and effects on the environment. These reveal pharmaceuticals as continuous emerging pollutants. Unlike pesticides and other priority pollutants, the behavior of pharmaceuticals in the environment has not been studied extensively (Díaz-Cruz et al. 2003). As discussed above, the widespread use of some drugs and their generally inefficient removal from WWTPs are the main reasons for the frequent detection of pharmaceuticals in aquatic bodies. Therefore, there is a growing need for alternative wastewater treatment processes for removing pharmaceuticals from waters. Recently, CWs have started to be researched for the removal of organic micro pollutants. However, this research has been focused mainly on human pharmaceuticals.

Veterinary pharmaceuticals are extensively used as the human ones but from an environmental point of view, veterinary drugs release and effects are less studied. Therefore, more research on these drugs is in need, namely on their detection in wastewaters, on evaluation of their release from WWTPs and on alternative wastewater treatment processes for their removal/elimination from livestock wastewaters.

This PhD project had two complementary aims: (1) analytical methodology optimization for determination of veterinary drugs; and (2) investigation of different factors that promote degradation/remediation of those drugs. For this purpose, the development of suitable analytical methodologies for a few selected drugs was firstly pursued using different techniques and applied for different environmental matrices from water to solids. Secondly, research was performed using activated sludge reactors to understand the behavior of the selected drugs in conventional wastewater treatment processes and confirm the environmental problematic. Finally, the research of CWs suitability for the removal of veterinary drugs from livestock wastewaters was evaluated.

1.9 Organization of the dissertation

This dissertation is structured in four parts.

The first part - Introduction - includes a background description of the environmental problematic, literature knowledge and research needs about the target contaminants and

principles of analytical methodology and water treatment technologies. The aims of the work are also presented, in addition to the present description of the organization of the Dissertation.

The second part - Experimental Section - contains aspects regarding the experimental planning and execution, namely the experimental design, materials, reagents and solutions used, sampling, analytical procedures and data handling methods.

The third part - Results and Discussion – contains the results of each one of the studies carried out, their interpretation and main conclusions. Each chapter starts with an introductory section, in which only specific aspects concerning the described work are focused and the specific aims are pointed out. When necessary, an experimental section devoted to particular experimental details is included. This third part embraces six chapters. Chapter 3 is devoted to the research on GC applicability for the determination of veterinary drugs. Chapter 4 is dedicated to the development of an expeditious analytical technique by SPE-HPLC for the analysis of veterinary drugs in wastewater samples, followed by analysis of real samples. Chapter 5 presents the optimization and validation of a multi-residue method for the analysis of veterinary pharmaceutical compounds in sludge and sediment samples. In Chapter 6 activated sludge systems removal efficiency of selected veterinary pharmaceuticals from slaughterhouse wastewater was researched. Chapter 7 is devoted to the investigation of the potential of *P. australis* for the removal of veterinary pharmaceuticals from aquatic media at microcosm-scale. Chapter 8 in continuation of Chapter 7 is dedicated to the investigation, also at a microcosm-scale, of CWs planted with *P. australis* for the removal of veterinary pharmaceuticals from livestock wastewater.

Chapter 4 is based on a paper published in Water Science and Technology and Chapter 7 as been reviewed as a short communication and published in Bioresource Technology. Chapter 6 and 8 are in submission process for publication in international peer review scientific periodicals.

The fourth part – Conclusions (Chapter 9) – contains the global conclusions and remarks of the work, as well as some considerations on future research needs.

A list of references is included at the end of each chapter.

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PART II

EXPERIMENTAL SECTION

Chapter 2 - Materials and Methods

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References

2.1 Material and reagents

Methanol and acetonitrile (CHROMASOLV®, HPLC grade), formic acid (98 %, reagent ACS) and hydrochloric acid (37 %, reagent ACS) were purchased from Sigma-Aldrich (Barcelona, Spain). High purity grade (>90 %) TET, ENR and CEF were supplied by Sigma-Aldrich (Barcelona, Spain). Other solvents and reagents used were of analytical grade. Nitrogen (99.995 % of purity) was from Air Liquide (Madrid, Spain).

Individual standard solutions of each pharmaceutical (1000 mg L⁻¹) were prepared in methanol. Weekly, a standard working solution of the mixture of all compounds at concentration of 40 mg L⁻¹ was prepared in methanol. This solution was used to prepare daily calibration standards solutions in deionized water (conductivity < 0.1 µS cm⁻¹). Individual standard working solutions, when needed along the different experiments, were also diluted in methanol. All standard solutions were kept in amber glasses at -20 °C in a refrigerator (light protected from photo-degradation).

For decontamination purposes all plastics and glassware were rinsed with soap, water, soaked overnight in 20 % (v/v) nitric acid (Pronalab), rinsed with water again and dried in a clean oven (40 °C).

2.2 Analytical methods

2.2.1 Methodology for veterinary drugs analysis in aqueous samples

A simple analytical method with solid-phase extraction (SPE) followed by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) for veterinary drugs analysis in treated wastewater samples was developed (Chapter 4). This methodology was further used along the PhD project to analyze veterinary pharmaceuticals not only in wastewaters (Chapter 4, 6, 7, and 8) but also in solid samples extracts (Chapter 5, 6 and 8) and in nutrient solution aqueous samples (Chapter 6 and 7).

SPE pre-treatment

In the final optimized conditions SPE cartridges, Oasis HLB (60 mg, 3 mL) cartridges from Waters Corporation (Millford, MA, USA), were firstly conditioned with 5 mL of methanol followed by 5 mL of deionized water. Samples (pH adjusted to 2) were passed through the pre-conditioned cartridges using a vacuum manifold system (Supelco, Spain) connected to a vacuum pump. The loaded cartridges were rinsed with 5 mL of a methanol/water mixture (5:95 v/v), and were dried under vacuum conditions for 30 min. The elution was performed with 5

mL of a methanol/formic acid mixture (96:4 v/v). The sample extracts were then evaporated to dryness under a nitrogen stream at 35 °C. The residues were dissolved in 1.0 mL of the HPLC mobile phase (water/formic acid, 99.9:0.1, v/v).

High-performance liquid chromatography conditions (HPLC)

The HPLC, which is a technique available in a large number of laboratories, was selected for this work. Separation was performed using a Beckman Coulter equipment (HPLC-system gold) provided with a diode array detector (DAD) (module 128) and an automatic sampler (module 508). The analytes were separated on a 150 mm × 4.6 mm C18 Luna column (Phenomenex, UK).

In the optimized experimental conditions a linear gradient program was used as follows: 100 % of eluent A (water-formic acid, 99.9:0.1, v/v), keeping isocratic conditions for 2 min, followed by a 10 min linear gradient to 70 % of eluent A (and 30 % of eluent B (acetonitrile)). Finally, initial conditions (100 % of eluent A) were reached again in 10 min, with a re-equilibration time of 2 min in order to restore the column. Flow rate gradient started with 1.0 mL min⁻¹, which was maintained for 2 min, followed by a 10 min flow rate gradient of 0.8 mL min⁻¹ and 10 min flow rate gradient of 1.0 mL min⁻¹. The sample injection volume was set at 50 µL and the detector signal was monitored at $\lambda = 280$ nm.

All HPLC solutions were filtered through 0.45 µm pore size membranes and degassed in an ultrasonic bath before use.

2.2.2 Methodology for veterinary drugs analysis in solid samples

A suitable extraction procedure was researched to analyze the selected veterinary drugs in solid samples (Chapter 5). This methodology was further used along the PhD project to analyze the drugs level in sludge (Chapter 6) and in sediment (Chapter 8).

In the optimized operating conditions each sludge sample was sequentially extracted three times with 10 mL methanol/formic acid (96/4, v/v) using USE (each period of 15 min; Elma, Transsonic 460/H model (Singen, Germany)). After each extraction the sample was centrifuged (2500 rpm for 5 min; Centrifuge Mixtasel, Selecta (Barcelona, Spain)) and the supernatants collected and combined. Depending on the drug concentration the extracts were either directly analyzed or pre-concentrated by SPE. For direct analysis 3 mL of the homogenized extract were evaporated to dryness at 35 °C and the residue dissolved in 1.0 mL of the HPLC mobile phase (water/formic acid, 99.9:0.1, v/v). For SPE pre-concentration the homogenized extract was dissolved in water to attain an organic phase fraction lower than 5% and the final aqueous sample pH was adjusted to 2. The SPE procedure was then similar to the

one described previously for wastewater (section 2.2.1). Extracts either direct or after SPE step were analyzed by HPLC-DAD as described previously (section 2.2.1).

For sediment samples, in the optimized conditions each sample was sequentially extracted two times with 10 mL methanol/acetone (95/5, v/v) using USE (15 min). After each extraction the slurry sample was centrifuged (2500 rpm, for 5 min) and the supernatants collected and combined. Extracts were further directly analyzed. For that, 14 mL of the homogenized extract were evaporated to dryness at 35 °C. The residue was dissolved in 1.0 mL of the HPLC mobile phase (water/formic acid, 99.9:0.1, v/v) and further analyzed by HPLC-DAD as described previously (section 2.2.1).

2.2.3 Microorganisms enumeration

To estimate microbial abundance total cell counts (TCC) were enumerated by the 4',6'-diamidino-2-phenylindole (DAPI) direct count method (Kepner Jr and Pratt 1994, Porter and Feyg 1980). For that, 1.5 mL of aqueous sample were added to 2.5 mL of saline solution (0.2 mm-filtered, 9 g L⁻¹ NaCl) with two drops of Tween 80 (0.2 mm-filtered, 12.5% (v/v)). Samples were then stained with DAPI and incubated in the dark for 12 min. Samples were filtered onto black Nucleopore polycarbonate filters (0.2 mm pore size, 25 mm diameter, Whatman, UK) under gentle vacuum and washed with autoclaved 0.2 mm-filtered distilled water. Membranes were set up in glass slides and cells counted at 1875x on an epifluorescence microscope (Laphot, Nikon, Japan).

2.2.4 Plant leaves chlorophyll content

Plant endurance was evaluated by determining chlorophyll contents (chlorophyll a, b, and total chlorophyll) and carotenoids in plant leaves, accordingly to (Abadía et al. 1984). Briefly, 1 g of fresh plant material was extracted with 25 mL of methanol and 0.1 g of CaCO₃. When the material was white due to the loss of the pigments (approximately 48 h), the solution was diluted in deionized water and absorbance measured at 663nm, 645nm and 480 nm.

2.2.5 Other determinations

Chemical oxygen demand (COD) and total and volatile suspended solids (TSS and VSS) were measured as described in Standard Methods (APHA-AWWA-WPCF 1992).

Toxicity in wastewater and sediment elutriate samples was measured using the test ToxScreen, a bioassay that uses a highly sensitive variant of the luminescent bacterium

Photobacterium leiognathi (Ulitzur et al., 2002). The 30 min assay was carried out using the pro-organic buffer according to the manufacturer instructions. Toxicity was estimated through the bacterial luminescence of the sample relatively to the test control, and was expressed as relative activity (%). Wastewater samples were centrifuged (15 min at 2500 rpm) prior to being processed. Sediment elutriates were prepared by agitation (200 rpm) of 2 g of sediment with 8 mL of deionized water followed by centrifugation (30 min at 2500 rpm). Toxicity was measured in terms of relative activity where 100 % activity represents 0 % toxicity.

2.3 Samples collection and preparation

2.3.1 Wastewater for methodology development and validation

The WWTPs effluents from two pig farms and two slaughterhouses and effluents from four urban WWTPs, all in the North of Portugal, (collected between November 2009 and February 2010) were used for SPE-HPLC method development and environmental levels research (reported in Chapter 4).

Urban WWTPs were all around big urban centers. Three urban WWTPs were based on tertiary treatment (URB WWTP 1, 2 and 3), whereas URB WWTP 4 just had secondary treatment. At least one of the urban WWTPs (URB WWTP 4) is known to receive treated wastewater from one of the slaughterhouses WWTP studied.

One of the slaughterhouse facilities (SH WWTP 5) had a capacity of 700 heads slaughtered per day, whereas the other one (SH WWTP 6) had a production of 200 heads slaughtered per week. SH WWTP 5 consisted of a solid-liquid separation pre-treatment facility followed by two activated sludge aerobic tanks (in series) and two biological decanters. SH WWTP 6 consisted of a solid-liquid separation pre-treatment facility followed by an aerobic lagoon with enzymes addition. The effluent from SH WWTP 6 is released into the URB WWTP 4.

Both pig farms WWTPs consisted of a solid-liquid separation pre-treatment facility followed by three lagoons in WWTP 7 (anaerobic, aerobic and polishing in reduced depth) and two lagoons in WWTP 8 (anaerobic and aerobic). The WWTP 7 had a capacity of 400 heads, whereas WWTP 8 had a capacity of 8000 heads.

Effluent grab samples were collected at the exit of the WWTPs (particularly, the effluents from pig farms and slaughterhouses WWTPs were collected at the exit of the last lagoon). The effluent collected was the one that was discharged into the environment (except effluent SH WWTP 6 that was released into URB WWTP 4). Samples were collected in amber glass bottles and immediately transported to the laboratory under refrigerated conditions.

At the lab all samples were filtered through 0.45 μm pore size membrane filters (Millipore, Ireland) to eliminate suspended solid matter, and then stored at 4 °C until sample analysis (within 1 week).

2.3.2 Solid samples for methodology development and validation

Sludge and sediment samples were collected to provide real environmental matrix for method development and validation (reported in Chapter 5).

Sludge samples were obtained from the municipal wastewater treatment plant URB WWTP 4 (see section 2.3.1) in October 2011. Samples were frozen at -20 °C until needed. Before analyses samples were thawed, centrifuged, to remove excess water, and then lyophilized in a Christ Alpha 1-4 freeze dryer (B. Braun Biotech International).

Sediment samples used were previously collected, by the workgroup in field campaigns along Portuguese natural estuaries. These samples were also frozen at -20 °C until needed, being lyophilized prior to use.

2.3.3 Sludge and wastewater for activated sludge batch reactors experiments

For experiments reported in Chapter 6, sludge was obtained from the municipal wastewater treatment plant URB WWTP 4 (see section 2.3.1) between March and October 2011. Experiments started within 18h of sludge collection, being the sludge kept aerated in the meanwhile.

Pre-treated wastewater was collected in the slaughterhouse SH WWTP 6 (see section 2.3.1) at the same day as sludge for wastewater batch experiments in October 2011.

Prior to use, sludge and wastewater were always homogenized, each in large (50L) open containers.

2.3.4 Plants and wastewater for *Phragmites australis* microcosm experiments

For studies reported in Chapter 7, *P. australis* with shoots were collected in *Rio Lima* margins (North of Portugal) in July 2010 (ENR trial), November 2010 (CEF trial), February 2011 (TET trial) and May 2011 (Wastewater trial) (Figure 4).

In the lab the roots were thoroughly washed to remove any sediment particles attached to their surface. Roots were submersed for a short period (ca. 1 min) in bi-deionized water containing the antimicrobial agent Micropur® (active ingredient: silver ions), to stop microbial action and then rinsed in bi-deionized water again (ca. 30 s) to remove the agent. Plants were kept one day in nutrient solution and then placed in the flasks used for the experiments.

Swine wastewater was collected in WWTP 7 (see section 2.3.1) in May 2011 (Figure 5), at the day of the beginning of the experiments.

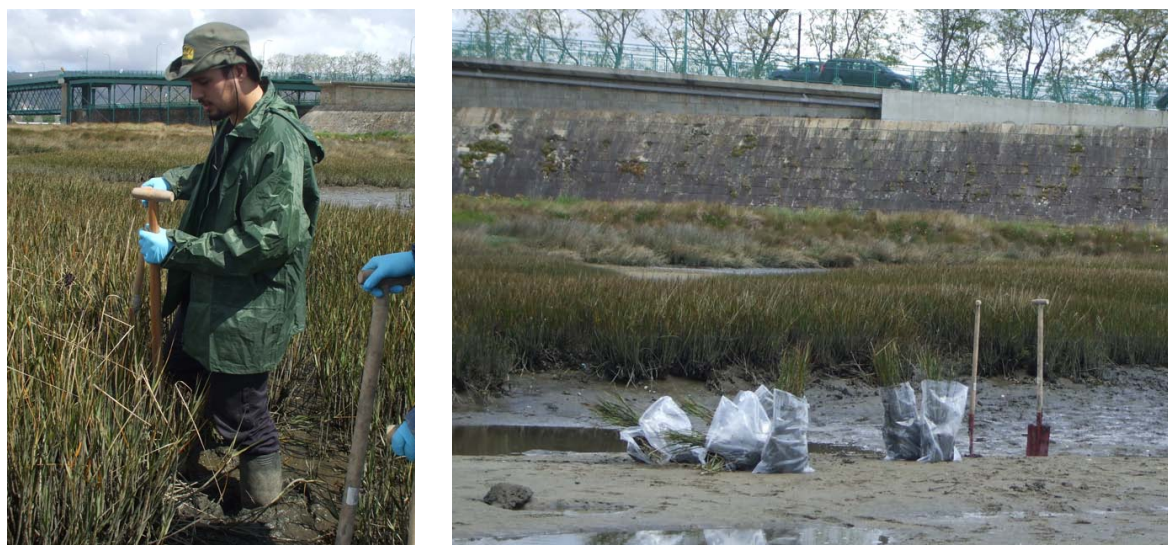


Figure 4 Plants sampling.

2.3.5 Plants, support matrix and wastewater for CWs microcosm experiments

For studies reported in Chapter 8, *P. australis* with shoots and sediment involving its roots were collected at Lima river margins (North of Portugal) in April 2012. Sand was collected simultaneously in the river basin (within 1 m of plant stands).

At lab, sediment was separated from the plant roots, which were afterwards washed. This sediment was further mixed with the sand (in 1:1 proportion) and homogenized to prepare a more porous media for the roots' bed substrate in the CWs microcosms.

Plants and roots' bed substrate were placed in CWs microcosms in the day of collection.

Treated wastewater was collected in a pig farm (WWTP 8, see section 2.3.1) weekly. Wastewater was introduced in the CWs microcosms at the day of collection.



Figure 5 Wastewater sampling at the wastewater treatment plant WWTP 7.

2.4 Sludge reactors and CWs studies experimental layout

2.4.1 Activated sludge batch reactors experiments

A series of laboratory-controlled experiments were carried out on both (i) synthetic medium (nutrient solution) and (ii) real slaughterhouse wastewater to evaluate the efficiency of a conventional wastewater treatment process (activated sludge), used for the treatment of wastewater, for the removal of three commonly used veterinary pharmaceuticals (ENR, CEF and TET) (studies reported in Chapter 6).

Batch experiments with nutrient solution

Batch assays were carried out in 5L poly(methyl methacrylate) cylinder reactors with bottom aeration (18h of daily aeration) that ensured also system agitation (Figure 6). Assays were performed with activated sludge (2 g L^{-1} of volatile suspended solids) and 4 L of modified Hoagland nutrient solution (Hoagland and Arnon 1950) to which $100 \mu\text{g L}^{-1}$ of the selected drug was added. Veterinary drugs, ENR, CEF and TET, were tested in separated assays (each drug in triplicate). For TET two assays were carried out, one assay with reactors exposed to natural light and another with reactors in the dark to prevent TET known photodegradation. Control reactors without activated sludge (only with nutrient solution and drugs) to evaluate possible natural attenuation of the drugs in the system and reactors with activated sludge but without drugs (only activated sludge and nutrient solution) to evaluate the biomass activity without antibiotics, were also used.



Figure 6 Sludge reactors overview.

The reactors were sampled regularly at the beginning (T_0) and at days 0.5, 1, 3, 6 and 10 for pH, VSS, TSS, microorganisms and veterinary drugs determination. All samples for drug analysis (250 mL per sample) were immediately acidified to pH 2 and frozen. Prior to veterinary drugs determination samples were thawed and centrifuged to separate the liquid fraction from the sludge. Liquid fraction was filtered through 0.45 μm pore size membrane filters (Millipore, Ireland) to eliminate suspended solid matter and further processed as described in section 2.2.1. Sludge fraction was lyophilized and further processed as described in section 2.2.2. After the 10th day sampling the reactors remained with 64% of the initial volume.

The set of reactors was kept in an open indoor environment subject to room temperature variations (17 – 22 °C) and natural day/night light exposure, except the few TET reactors that were kept in the dark

Batch experiments with wastewater

Batch assays were carried out in the same conditions as experiments with nutrient solution. Veterinary drugs, ENR and TET, were tested individually in independent assays with an initial concentration level of 100 $\mu\text{g L}^{-1}$. Control reactors without activated sludge (only with wastewater and drugs) to evaluate possible natural attenuation of the drugs in the system and with activated sludge but without drugs (only activated sludge and wastewater) to evaluate the biomass activity without antibiotics, were also used.

The reactors were sampled regularly at the beginning (T_0) and at days 0.5, 1, 3, 6 and 10 for pH, VSS, TSS, microorganisms and veterinary drugs determination. For drug determination 100 mL (in triplicate) were taken from each reactor. All samples were treated and analyzed as described above. After the 10th day sampling the reactors remained with 55% of the initial volume.

The set of reactors was kept in similar conditions in the same open indoor environment described above.

2.4.2 *Phragmites australis* microcosm experiments

Two microcosm-scale studies, one in a synthetic medium (nutrient solution) and another in livestock wastewater were carried out to access the capacity of *P. australis* to remove the three veterinary pharmaceuticals ENR, CEF and TET from aquatic mediums (studies reported in Chapter 7).

Nutrient solution experiments

Plants were placed in 300 mL flasks (3 plants by flask) with 250 mL modified Hoagland nutrient solution (Hoagland and Arnon 1950) and an appropriate amount of the drug under

study (Figure 7). Control flasks with drug and nutrient solution but without plants were also used to evaluate possible drugs natural degradation.

For each drug (ENR, CEF and TET), two levels of concentration were tested, 10 and 100 $\mu\text{g L}^{-1}$, along a 7 days period.



Figure 7 Plants microcosms' overview - nutrient solution experiments.

Flasks were doped at day 0, 2, 4 and 6 making the concentrations during the study range from 10 to 40 $\mu\text{g L}^{-1}$ and from 100 to 400 $\mu\text{g L}^{-1}$ at the begin and end of the experiment, respectively. Flasks of solutions without plant (control) and of solutions with plants (SWP) were collected in triplicate at day 1, 4 and 7. Water evaporation was controlled by addition of deionized water, when needed, to ensure a final 250 mL sample. Additionally, after liquid sample collection, each set of 3 plants was placed again in a new flask with new nutrient solution (250 mL, without drug addition) for a period of 2 days to study possible plant exudation of the drugs. All samples were frozen until 24h prior to being processed for drug analysis (Section 2.2.1).

The set of flasks for the 7 days study period was kept in an open indoor environment (outside the laboratory, in an open area inside the Faculty building), subject to environmental temperature variations and environmental light exposure. ENR experiment took place during July 2010 with an environment temperature ranging from 24 (night) to 30 °C (day). CEF experiment took place during November 2010 with an environment temperature ranging from 12 (night) to 18 °C (day). TET experiment took place during February 2010 with an environment temperature ranging from 16 (night) to 22 °C (day). The pH of the solutions was also monitored during the assays.

Wastewater experiments

Plants were placed in 300 mL flasks (4 plants by flask) with 250 mL of treated swine wastewater (see section 2.3.3) and the selected drug (Figure 8). For each drug (ENR, CEF and TET), one level of concentration, $100 \mu\text{g L}^{-1}$, was studied along a 7 days period. Flasks were doped only at the beginning of the experiment. Control flasks with the same volume of wastewater but without plants, were used to evaluate drugs possible natural degradation. Control flasks without veterinary drugs, either with plants and wastewater or with plants and nutrient solution were also used, to evaluate plants endurance along the study. Control flasks only with wastewater for microorganism enumeration along the study were also used. Solutions of all flasks were collected in triplicate at day 7.



Figure 8 Plants microcosms' overview - wastewater experiments.

Additionally, after liquid sample collection, each set of the 4 plants roots was shaken in a new flask with de-ionized water (250 mL) during 2 min for cleaning. This solution was also stored to check for possible adsorption of drugs to plant roots. Then, each set of plants was placed again in wastewater (250 mL, without drug addition) for 2 days to study possible exudation of drugs from plants.

All liquid samples for drug analysis were frozen until 24h prior to being filtered through $0.45 \mu\text{m}$ pore size membrane filters (Millipore, Ireland), to eliminate suspended solid matter, and further processed for veterinary drugs determination (Section 2.2.1).

The set of flasks for the 7 days study period was kept in the same place than that of the

nutrient solutions trial, previously described. Experiment took place during May 2010 with an environment temperature ranging 17 (night) and 26 °C (day). The pH of the samples was monitored during the assays.

2.4.3 CWs microcosm experiments

A microcosm-scale study was carried out to evaluate the capacity of CWs planted with *P. australis* to remove ENR and TET from wastewater (studies reported in Chapter 8).

Microcosm's setup

Sixteen microcosms were set up using plastic containers (0.4 m x 0.3 m x 0.3 m) filled with a first layer of gravel (4 cm depth), a second layer of lava rock (2 cm depth) and finally the roots' bed substrate obtained as described in section 2.3.5 (10 cm depth) reaching a total depth of 16 cm. Water level was maintained just above the substrate surface, corresponding to a flooding rate of approximately 100%. The systems were designed to operate in a batch mode, i.e. with the initial load of water and without any running flow during the assays, having only a tap at the base for sample collection. All microcosms were wrapped in aluminum foil to simulate a real system (where there is no light penetration at substrate level) and prevent the occurrence of photodegradation of the compounds under study (Figure 9).



Figure 9 Constructed wetland (CW) microcosm setup.

Seven microcosms were planted with *P. australis* (section 2.3.5) (density of ca. 40 plants per microcosm). Another nine microcosms were left unplanted. In two of the unplanted microcosms the substrate was sterilized (a 0.7 mol L⁻¹ ZnCl₂ solution (Teixeira et al. 2012) was circulated along the first conditioning week, see below), to study the drug adsorption capacity of the support matrix of the microcosms. In addition to these sixteen microcosms, two additional unplanted microcosms (one for each drug) were used just filled with mixed gravel and lava rock (reaching the same total depth of 16 cm) to study possible differences in treatments with and without the roots' bed substrate.

The set of microcosms was kept in an open indoor environment (outside the laboratory, in an open area inside the Faculty building), subject to environmental temperature variations and environmental light exposure. Over the thirteen weeks (April to July), in which the microcosms were set-up and running, minimum temperatures were 16 ± 2 °C and maximum 28 ± 8 °C.

Microcosms operation and sampling

Microcosms were left to acclimate for one week. During this period a modified Hoagland nutrient solution (Hoagland and Arnon 1950) was added (replaced daily) to maintain plants at optimum nutritional conditions. The solution was added to all microcosms, including unplanted ones, to ensure that support matrixes of all microcosms were in the same conditions.

Thereafter, three sets were run in parallel (Figure 10), two with wastewater doped with one of the two drugs tested (ENR or TET) and a third set only with wastewater for control purposes. For each drug three planted microcosms, three unplanted and one sterilized were used. For each drug a 100 µg L⁻¹ level of concentration was tested. The wastewater was kept in the microcosms for one week. The microcosms were manually operated for a daily recycle of the wastewater to prevent the development of anoxic areas within the support matrix. The experiment was prolonged for twelve 1-week cycles to evaluate treatment efficiency and monitor plant stress, microbial community and support matrix adsorption capacity. For that purpose, each week, the microcosms were completely drained and refilled with new-doped wastewater. Water evaporation was controlled by addition of deionized water.

Water pH was monitored along the experiment (at the end of each one-week period). Plant endurance was accessed through plant leaves chlorophyll content determination. For that purpose five leaves per microcosm were collected, sliced, homogenized and further processed as described above (Section 2.2.4).

Aqueous and solid samples were collected from ENR, TET and control microcosms, at week 1, 2, 4, 8 and 12 from the planted systems and only at week 1, 2 and 4 from the unplanted ones. The unplanted systems clogged at week 6 and became impracticable to be operated until the 12 week.

Liquid samples (250 mL per microcosm) were collected, acidified with hydrochloric

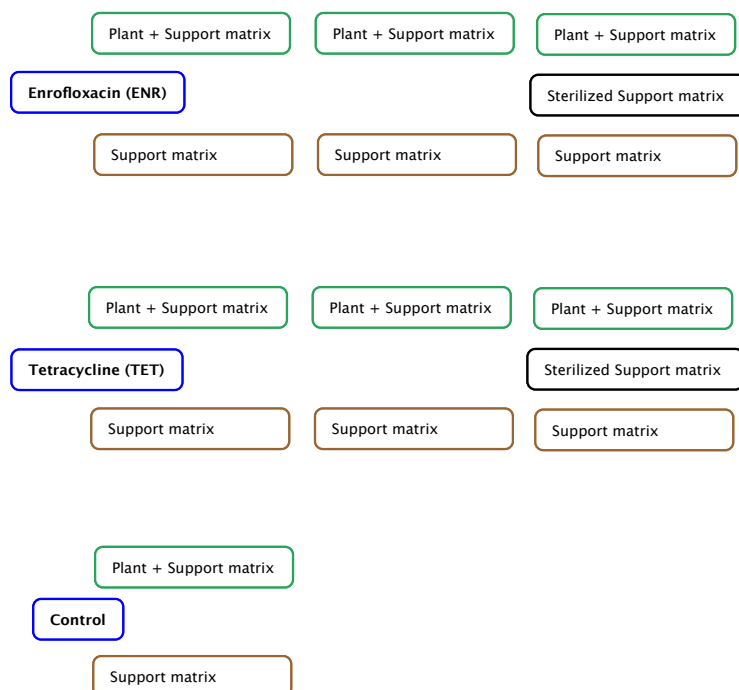


Figure 10 Constructed wetlands (CWs) microcosm experiment setup diagram.

acid and kept at -20°C until 24h prior to being filtered through $0.45\ \mu\text{m}$ pore size membrane filters (Millipore, Ireland), to eliminate suspended solid matter. Afterwards samples were processed for veterinary drugs determination (Section 2.2.1). Bed root substrates portions were collected in each microcosm in six points to obtain composite samples. These samples were then lyophilized, sieved and kept at -20°C until further analysis for veterinary drugs determination (Section 2.2.2).

Additionally, 10 mL of liquid samples of each microcosm were also collected and immediately stored at -20°C until further processed for toxicity screening. Toxicity in wastewater and sediment elutriate samples (except those from sterilized microcosms) was measured using the test ToxScreen (Section 2.2.5).

2.5 Statistical analysis

Statistically significant differences among samples for 5% level of significance were evaluated through ANOVA tests using GraphPad Prism software and Tukey pairwise comparisons.

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PART III

RESULTS AND DISCUSSION

Chapter 3 - Is gas chromatography suitable for the analysis of veterinary drugs?

3.1 Introduction

3.2 Experimental layout

3.2.1 Reagents and solutions

3.2.2 Chromatographic conditions

3.2.3 Procedures

3.3 Results and discussion

3.4 Conclusions

References

3.1 Introduction

A wide variety of techniques have been used for the analysis of broad therapeutic classes of drugs consumed worldwide as mentioned in Chapter 1. Nowadays, GC with MS can still be the perfect technique for certain classes of pharmaceuticals, being an alternative to HPLC and LC coupled to a MS detector (Buchberger 2007). In fact, despite the majority of pharmaceuticals lacks sufficient volatility to be directly analyzed by GC, various groups of pharmaceuticals can be derivatized making them suited for GC analysis (Buchberger 2007). When efficient derivatization is achieved, GC methods, especially GC-MS applications, are powerful tools for the determination of pharmaceuticals in the ng or µg per litter levels. Various derivatizing agents can be used for this purpose, but one should be aware that the derivatization step in any case can influence the accuracy of the method, as losses of analytes can occur or the derivatization reaction can be incomplete (Kostopoulou and Nikolaou 2008).

The SPME, combined with GC, allows the derivatization of the analytes to take place in the sample matrix, in the SPME fiber coating or in the GC injector port, thus allowing an *in situ* derivatization followed by GC analysis and presenting a rapid and efficient method of analysis (Araujo et al. 2008, Lamas et al. 2004). Few studies have considered the option of performing the derivatization directly in the water matrix. For instance, Araujo et. al. (2008) have tested methylation of non-steroidal anti-inflammatory drugs where acidic drugs are converted to less polar methyl esters, which may improve the extraction into the SPME fiber.

The SPME has been used in association either with GC, with or without derivatization (Araujo et al. 2008, Huppert et al. 1998), or with HPLC/LC-MS (Lock et al. 1999, Mitani and Kataoka 2006) for pharmaceuticals analyses. However, as further our knowledge, application of SPME-GC-MS with derivatization for veterinary drugs has not been reported yet. Therefore, and considering the previous experience of work with this technique (an expeditious technique which generally allows attaining lower LODs than traditional GC-MS analysis minimizing matrix interferences) a survey of the possibility of using SPME-GC-MS for analysis of some veterinary drugs very much used in livestock industry: ivermectin (IVE), penicillin G (PEN G) and V (PEN V), ENR and CEF was carried out. The selected veterinary drugs have a higher molecular mass than those of human pharmaceuticals that were already analyzed by SPME-GC-MS (Araujo et al. 2008, Huppert et al. 1998), but some of them have similar functional groups that could suffer the same reaction with the derivatization agent.

3.2 Experimental layout

3.2.1 Reagents and solutions

Ethanol absolute for HPLC, was obtained from Panreac (Barcelona, Spain). The

derivatizing reagents acetic anhydride (AA) (purity 98 %), ethyl chloroformate (ECF) (purity 98 %) and dimethyl sulphate (DMS) (purity 99.8 %) were obtained from Merck (Darmstadt, Germany), Fluka (Darmstadt, Germany) and Sigma-Aldrich (Darmstadt, Germany) respectively. Ion pairing reagent tetrabutylammonium bisulphate (TBA-HSO₄) (purity 99 %) was obtained from Fluka. Flufenamic acid (FLU) (purum), ibuprofen (IBU) (purity 98 %), and ivermectin (IVE) (purum) from Sigma, penicillin G (PEN G) (purity 99.7 %) from Riedel-de Haën (Hanover, Germany) and enrofloxacin (ENR) (purity 98 %), penicillin V (PEN V) (purity 98.3%) and ceftiofur (CEF) (purity 99.4 %) from Fluka were used to prepare stock standard solutions in ethanol (1 g L⁻¹), being stored at 4 °C. All individual working standard solutions were prepared from these stock solutions in ethanol.

3.2.2 Chromatographic conditions

The GC-MS determinations were performed using a Varian Saturn 2000 mass spectrometer (Walnut Creek, CA) coupled to a Varian 3900 gas chromatograph equipped with a split/splitless injector port, a SPME liner (0.75 mm ID), a microseal septum system (Merlin, Half Moon Bay, CA) and a CP-Sil 8CB Low Bleed/MS (Varian) column (60 m length x 0.25 mm diameter, 0.25 µm film thickness). The carrier gas was helium of high purity (99.9995% from Air Liquide) at a constant flow of 1.0 mL min⁻¹. The Varian computer software MS Workstation 6.30 controlled the GC-MS.

Analyses by GC coupled to a flame ionization detector (FID) were performed using a Varian CP-3800 gas chromatograph equipped with a split/splitless injector port 1079, a liner (3.4 mm ID) and a VF-5ht column (30 m length x 0.32 mm diameter, 0.10 µm film thickness). The carrier gas was helium of high purity (99.9995% from Air Liquide) at a constant flow of 2.0 mL min⁻¹. The other gases used, nitrogen (25 mL min⁻¹), hydrogen (30 mL min⁻¹) and air (300 mL min⁻¹) (all at least 99.999%), were also from Air Liquide. The Varian computer software Star Chromatography Workstation v6.41 controlled the GC-FID.

The GC conditions used were adapted from (Araujo et al. 2008) according to the available technical conditions. The injector temperature was set at 250 °C and the oven temperature was held at 50 °C for 3 min, then heated up at a 30 °C min⁻¹ rate to 300 °C and held at this temperature for 5 min. The mass spectrometer was operated in full scan mode, using library search (m/z = 40 - 600), with the temperatures of the transfer line and of the ion trap set as 230 °C and 200 °C, respectively. FID detection was carried out with the detector oven at 320 °C.

3.2.3 Procedures

In all the tests, three different concentrations, between 0.5 and 5 mg L⁻¹, of each compound were used. Adequate volumes of each working standard solution and water were placed in the 20 mL screw-cap amber glass vial in which the SPME extraction was carried out. Final volume of the solution was 10 mL for headspace SPME or 18 mL for immersion SPME. The derivatization reagent used in each case conditioned the addition of other reagents to the vial (Araujo et al. 2008, Van Hoeck et al. 2009).

For DMS, phosphate buffer solution (pH 6.0, 0.5 mol L⁻¹, 3:10 (v/v)) and solid Na₂SO₄ (4:10 (m/v)) were added to the vials and then, prior to closure of the vial, the ion-pairing reagent (TBA-HSO₄, 0.1 M, 1:100) was also added. For ECF, ethanol (3:100 (v/v)) was added prior to closure of the vial. For AA, Na₂CO₃ (1:20 (m/v)) was added prior to closure of the vial.

All the derivatizing agents were added through the cap septum (1:100 (v/v) for DMS and ECF or 1:20 (v/v) for AA), at the end of the respective vials preparation, with the vials already sealed to prevent the loss of volatile species that could form during the reaction.

The SPME (either in headspace or in immersion) was performed using an autosampler Combi Pal model, CTC Analytics (Zwingen, Switzerland) that controlled extraction temperature, time and agitation of the vials. Polydimethylsiloxane (PDMS), polyethylene glycol (PEG) and polyacrylate (PA) fibers from Supelco (Bellefonte, PA, USA) were tested. SPME conditions are detailed in Table 2. After the extraction, the fiber was directly exposed to the hot injector of the GC for 3 min (split off) and the chromatogram was registered.

Table 2 Solid-phase microextraction (SPME) conditions

Extraction mode	Headspace (HS) or Immersion (IM)
Fiber type	100 μ m film thickness Polydimethylsiloxane (PDMS) 60 μ m film thickness Polyethylene glycol (PEG) 85 μ m film thickness Polyacrylate (PA)
Pre-incubation time	10 minutes
Pre-incubation temperature	70 °C
Pre-incubation rotation	500 rpm ^a
Extraction time	45 minutes
Extraction temperature	70 °C
Extraction rotation	250 rpm ^a

^a rotation per minute

For the studies using the GC-FID, direct injection of 1 μ L of solution was performed. The solutions tested were blanks (ethanol or ethanol plus the derivatizing agent), each standard (1 mg L⁻¹) and each standard with derivatizing agent (1:1 of standard to derivatizing agent (v/v)).

3.3 Results and discussion

Before initiating the tests with the selected veterinary drugs, the method adapted from (Araujo et al. 2008) was successfully applied for the determination of a mixture of IBU and FLU, two human drugs. The reported method was reproduced in the laboratory with the available equipment. The derivatizing agent used was DMS and the SPME was carried using a PDMS fiber in headspace. Some modifications were needed, like adjustment of the total volume of the solutions for the available vials and also the adaptation of the headspace SPME conditions from a manual procedure to the automatic sampling system. MS workstation software allowed the identification of IBU and FLU by selection of specific ions from the obtained chromatograms and comparison with the mass-to-charge ratio of the most abundant ions from the respective derivatized compounds (Figure 11).

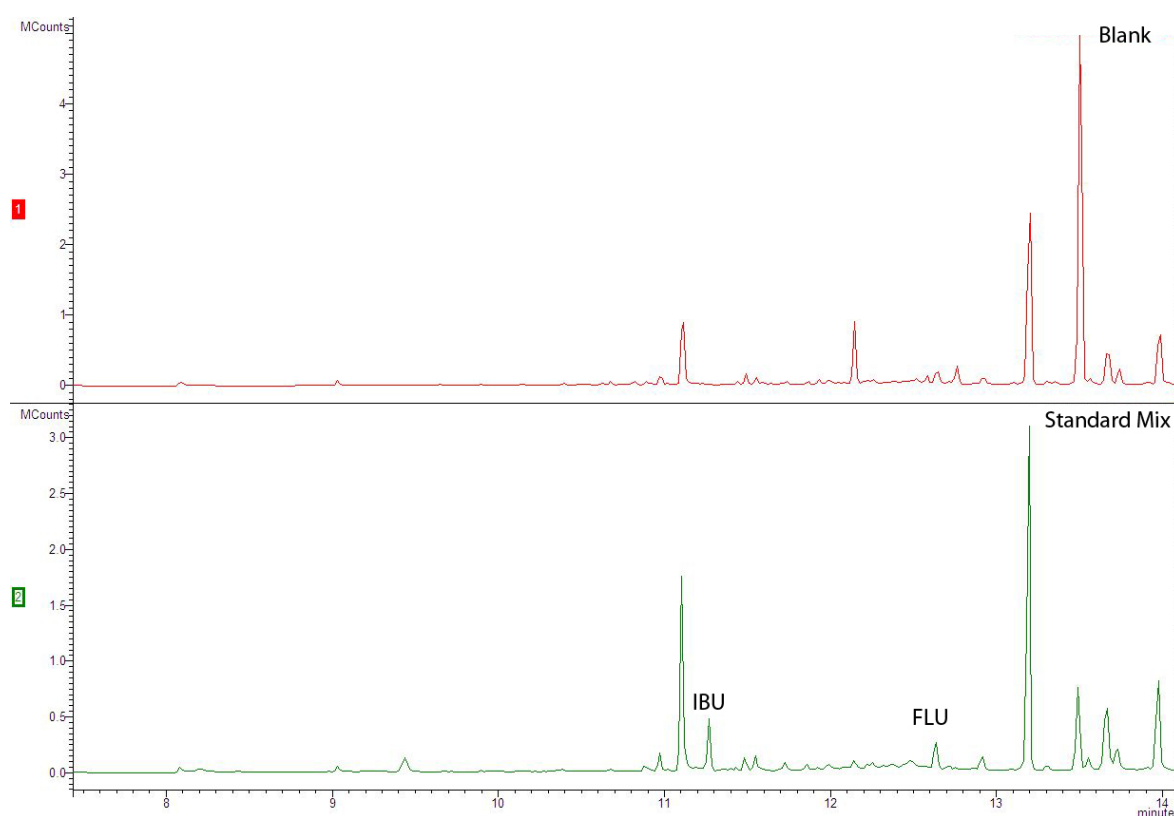


Figure 11 Chromatograms of headspace solid-phase microextraction (SPME) followed by gas chromatography (GC) coupled to mass spectrometry (MS) analysis of aqueous ibuprofen (IBU) and flufenamic acid (FLU) mixed standard ($10 \mu\text{g L}^{-1}$) derivatized by dimethyl sulphate (DMS) and respective blank solution.

Veterinary drugs tests started with the analysis of individual standards of each one of the selected drugs in the same conditions, as those used before for IBU and FLU. The veterinary drugs have a higher molecular mass than those of IBU and FLU, but some of them have similar functional groups. The GC time window was increased (from 15 to 45 min) to ensure enough analysis time and an efficient cleaning of the GC column at the end of the run.

Chromatograms presented several peaks, some identified by the software, however none was identified as resultant from the compounds under study. In Figure 12, an example is shown. Comparison of chromatograms of the derivatized compounds at different concentrations did not reveal differences neither between them nor from the common matrix. Therefore, none of the peaks observed in the different chromatograms could be attributed to the compounds under study.

So, several changes of different parameters were carried out. When performing GC

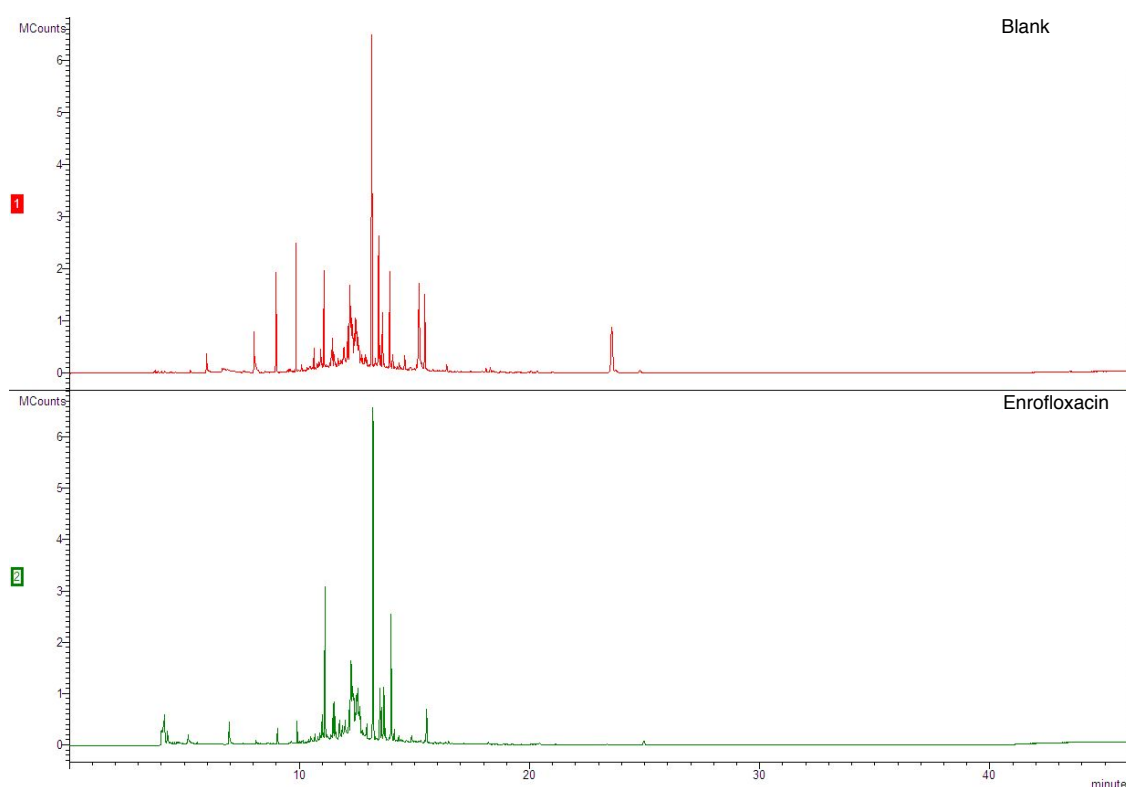


Figure 12 Chromatograms of headspace solid-phase microextraction (SPME) followed by gas chromatography (GC) coupled to mass spectrometry (MS) analysis of aqueous enrofloxacin standard (0.5 mg L^{-1}) derivatized by dimethyl sulphate (DMS) and respective blank solution.

analysis the compounds under study need to be volatilized in the GC injector port. For the veterinary drugs under study there is a lack of information on derivatization reactions, namely *in situ* derivatization with simultaneous SPME. Admitting that DMS was not a suitable derivatizing agent in the present case (despite the selected veterinary drugs have some similar functional groups to those of IBU and FLU), two other derivatizing agents, ECF and AA, were tested. ECF has been used before (Van Hoeck et al. 2009) for the derivatization of acidic and amine based compounds and AA has been used by the same authors for phenolic compounds, and some of those functional groups are also present in the veterinary drugs under study. In addition, three types of fibers (PDMS, which is apolar, and two polar ones PEG and PA), and two exposure procedures (headspace and immersion) were used for the SPME step.

Each drug individually combined with a derivatizing agent and with each type of SPME fiber and exposure mode was analyzed.

However, results were not positive in any case (additional exemplificative figures for all the drugs with the different derivatizing agents using headspace SPME PDMS fiber may be found in appendix A1). For ECF no peaks could be differentiated from the blank. For the AA derivatizing agent chromatograms were different from the blank but similar for the different individual standards at different concentrations. The software could not identify the compounds responsible for the questionable peaks. Therefore, none of the compounds could be identified with any of the derivatizing agents or any of the fibers tested in both exposure modes. The problems encountered may result of:

1) Inefficiency of the derivatizing agents, which could not transform the compounds into more volatile species. Nothing is known about application of derivatizing agents to the veterinary drugs under study. Although the derivatizing agents selected were used for compounds with similar functional groups to those under study there is no guarantee that the derivatization reaction occurred. In addition, even in the case of a successful derivatization, the new compounds may still not be volatilized in the GC injector port.

2) Inefficacy of adsorption of the derivatized compounds on the SPME fibers tested because the respective molecular mass was too high.

To try to understand the unsuccessful attempts for the veterinary drugs analysis, each drug (both human and veterinary) was analyzed by GC-FID. Direct injection of each drug as standards not derivatized, to check possible interferences from impurities (drug in ethanol solution), or derivatized (drug plus derivatizing agent) in the GC injection port was carried out. IBU and FLU, without or with derivatization by either DMS or ECF were successfully identified in the chromatograms, whereas the veterinary drugs were not. As expected, the non-derivatized veterinary drugs (with high boiling points) were not volatile due to their high molecular mass and intermolecular interactions. On the other hand, the derivatization agents used probably were also not suitable to transform the drugs into more volatile species.

However, no information on derivatization reactions and products of derivatization sufficiently volatile for GC determination of these veterinary drugs were found in the literature.

3.4 Conclusions

The set of results obtained in this work suggest that GC is not suitable for the analysis of the selected veterinary drugs.

Therefore, the unsuccessful application of headspace SPME combined with GC-MS, did not allow attaining the goal of reducing sample matrix interferences, which would provide a rapid and more efficient analytical tool.

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Chapter 4 - Simultaneous determination of several veterinary pharmaceuticals in effluents from urban, livestock and slaughterhouse wastewater treatment plants using a simple chromatographic method

4.1 Introduction

4.2 Experimental layout

4.2.1 Solid-phase extraction (SPE) pre-treatment

4.2.2 High-performance liquid chromatography (HPLC)

4.3 Results and discussion

4.3.1 Characteristics of the method

4.3.2 Survey of veterinary pharmaceuticals in WWTPs effluents

4.4 Conclusions

References

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4.1 Introduction

Effluents from WWTPs are considered one of the important sources of pharmaceutical residues to the environment (Halling-Sørensen et al. 1998, Hirsch et al. 1999, Kolpin et al. 2002, Watkinson et al. 2009) as discussed in Chapter 1. For veterinary drugs, inputs from manure application to soils and aquaculture activities have been considered the most important sources (Boxall et al. 2004). Nevertheless, recent research showed that direct discharges of livestock industries (Tong et al. 2009) or slaughterhouses effluents (Shao et al. 2009) into the aquatic environment can also be a significant source of these compounds in the environment. In addition, several of these industries have WWTPs to deal with their effluents, however these systems are built to treat organic, nitrogen and phosphorus loads, and are not designed to remove pharmaceutical compounds. Although some of these effluents are directed to urban WWTPs, most of them are directly discharged into the environment. Therefore, these WWTPs effluents must also be considered as a possible source of veterinary drugs and should be taken in consideration. Nevertheless, only a few works were devoted to the monitoring of these drugs in these types of effluents, mostly in Asian countries (Ben et al. 2008, Shao et al. 2009, Tagiri-Endo et al. 2009, Tong et al. 2009), in which concentrations as high as 400 $\mu\text{g L}^{-1}$ have been found (Tagiri-Endo et al. 2009), and more studies are in need.

This chapter presents the results of a survey of five veterinary antibiotics, minocycline (MNC), oxytetracycline (OTC), TET, ENR and CEF in Portuguese WWTPs effluents of both livestock and slaughterhouse as well as of urban WWTPs effluents. Surveys of veterinary antibiotics from Portuguese livestock, slaughterhouses or urban WWTPs effluents were not available in the literature. In addition, as far as we know, levels of ceftiofur have not been searched before in WWTPs effluents anywhere. Only one study for ceftiofur determination on surface water samples was available in the literature (Puig et al. 2007).

Although reviews about analytical methodologies for determination of pharmaceutical compounds in the aquatic environment are available in the literature (Fatta-Kassinos et al. 2011, Seifrtová et al. 2009, Speltini et al. 2010, Wong and MacLeod 2009) only a few studies on applications directed to effluents of livestock industries (Ben et al. 2008, Campagnolo et al. 2002, Malintan and Mohd 2006, Tagiri-Endo et al. 2009) and slaughterhouses (Shao et al. 2009) could be found. Those effluents normally have much higher organic contents than those from domestic wastewaters, which makes them more difficult to analyse. Most of the existing methods use solid-phase extraction (SPE), for matrix cleaning and analytes pre-concentration, and either LC-MS (Ben et al. 2008, Campagnolo et al. 2002) or LC-MS² (Shao et al. 2009, Tagiri-Endo et al. 2009, Tong et al. 2009). HPLC with different detectors, which is a primary tool of analysis in most service, commercial and research laboratories, was applied (SPE-HPLC with UV detection) (Malintan and Mohd 2006) to veterinary drugs in swine wastewater effluents, for determination of sulfonamides (one family of antibiotics). This accessible technique (although less sensitive and selective than LC-MS and LC-MS²) has the potential to be applied

to detect other veterinary drugs in these types of effluents. Therefore, in this work, for the simultaneous analysis of the five selected compounds, SPE combined with HPLC-DAD was used after a previous validation of the method.

4.2 Experimental layout

Material and reagents, as well as samples collection are detailed on Chapter 2. High purity grade (>90 %) MNC and OTC were supplied by Sigma–Aldrich (Barcelona, Spain).

4.2.1 Solid-phase extraction (SPE) pre-treatment

Oasis HLB (60 mg, 3 mL) cartridges from Waters Corporation (Millford, MA, USA) were used for SPE. The sorbent inside the cartridge is a hydrophilic and lipophilic polymer that can simultaneously extract acidic, neutral and basic polar analytes (Weigel et al. 2004). Suitable SPE extraction conditions were selected using firstly mixed aqueous standard solutions of the pharmaceuticals. The composition of the elution solution was varied, from pure methanol to methanol mixed with different percentages of formic acid.

The extraction efficiency of Oasis HLB cartridge was further evaluated with all types of wastewater samples (see section 2.3.1). Prior to SPE extraction, each filtered WWTPs effluent sample was spiked with stock mixed standard solution to evaluate the analytical methodology. Blanks (non-spiked water samples) were analyzed for all samples for control purposes. Effect of sample pH, adjusted to 2.0, 4.0 and 7.0 (by addition of concentrated hydrochloric acid) on the efficiency of the SPE procedure was evaluated for the different types of WWTP effluents tested.

Prior to SPE extraction, EDTA was added to each doped sample to test a possible positive influence in tetracyclines SPE recoveries.

Final optimized conditions are described on section 2.2.1.

4.2.2 High-performance liquid chromatography (HPLC)

The selection of the HPLC mobile phase and chromatographic conditions were adapted from those reported in previous works (Bueno et al. 2007, Castiglioni et al. 2005, Turiel et al. 2006, Yang et al. 2005) for human pharmaceuticals of different therapeutic groups (tetracyclines, sulfonamides, quinolones and fluoroquinolones).

The chromatographic conditions were firstly optimized, by using aqueous standard solutions of the selected veterinary compounds. To obtain chromatograms with a good resolution of the target compounds in a short analysis time several conditions, such as different eluents, different gradients as well as different detection wavelengths were tested.

Equipment used and final optimized conditions are described on section 2.2.1.

4.3 Results and discussion

4.3.1 Characteristics of the method

HPLC conditions established and described in the experimental chapter enabled efficient separation of the five selected veterinary drugs of different families in a single run of 24 min with relatively low consumption of acetonitrile.

Mixed aqueous standard solutions, covering the concentration interval from 0.1 to 20.0 mg L⁻¹ for each drug, were used to determine the characteristics of the analytical method (Table 3). Linear response range was found from 0.1 to 10 mg L⁻¹, with correlation coefficients (R^2) higher than 0.985 ($n = 8$). Slope of the linear regression presented an overall variation lower than 8 % for the different drugs along 18 days. The precision of the HPLC-DAD method was determined by the intra-day and inter-day variation of one mixed aqueous standard solution (5 mg L⁻¹), analyzed three times per day for five consecutive days. Repeatability (1.6 – 3.8 %) was lower than reproducibility (ca. 5.7 %) (Table 3), with relative standard deviations (RSD) of 6 % in all cases ($n=5$).

Table 3 High-performance liquid chromatography (HPLC) method characteristics determined using aqueous mixed standard solutions of the pharmaceutical minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF)

Pharmaceutical	Linear Range (mg L ⁻¹)	Slope Variation (%) (n=18)	R^{2a} (n=8)	Repeatability (%)	Reproducibility (%)	LOD ^b (mg L ⁻¹)	LOQ ^c (mg L ⁻¹)
MNC	0.1 - 10	5	0.993	2.0	5.6	0.1	0.3
OTC		7	0.985	2.2	5.9	0.1	0.3
TET		8	0.995	2.0	5.6	0.1	0.3
ENR		8	0.995	1.6	5.6	0.04	0.1
CEF		5	0.994	3.8	5.7	0.15	0.5

^a Linear correlation coefficient

^b Limit of detection

^c Limit of quantification

The low concentrations of pharmaceuticals in the aquatic environment in addition to the complex nature of the matrix of the WWTPs effluents, imply that the samples have to be submitted to a clean-up and pre-concentration step prior to further analysis (Buchberger 2007). Suitable composition of the SPE elution solution was chosen. It was observed that when methanol was used alone, drugs recoveries were below 50 % and better extraction conditions were obtained when formic acid was added to the elution solvent. Using methanol/formic acid mixture at 96:4 (v/v) proportion the average recovery of all pharmaceuticals, after SPE extraction, was higher than 90 % (results not shown). Accordingly, it has been previously shown (De Zan et al. 2008, Yang and Carlson 2003) that the presence of acids in the elution solvent could improve the pharmaceuticals recovery from the SPE cartridges.

Once the best methodological conditions were established for the overall SPE-HPLC-DAD procedure, method characteristics, namely limit of detection (LOD), limit of quantification (LOQ) and recovery, were determined using all WWTPs effluent samples.

The complex matrix of the wastewaters, especially those from livestock and slaughterhouse, may lead to blockage of the SPE cartridges if a high sample volume is passed through the cartridge. Therefore, experiments were carried out using only 50 mL of sample.

A possible matrix effect on HPLC drug quantification was firstly tested. For this purpose, using SPE pre-treated doped wastewater samples, a comparison of results obtained with standard addition method, with those obtained using external calibration with aqueous mixed standards solutions was carried out. As statistically significant differences were not found, daily external calibration with aqueous mixed standard solutions was further used, which is an advantage because it is much less time consuming.

The pH of the sample may affect drug SPE recoveries once the studied antibiotics are acids with different pKa values. Therefore tests were conducted to evaluate the effect of pH on the efficiency of the SPE procedure (Figure 13). Results indicated that the effect of pH depended on the drug itself. A pH of 2.0 was chosen as a compromise to attain the best extraction efficiency possible for the simultaneously extraction of all the drugs. At pH 4.0 oxytetracycline and tetracycline SPE recoveries were higher but those of ceftiofur and enrofloxacin were lower. In general, at pH 7.0 the recoveries for all the compounds were lower than at pH 2.0. Previous works reported that a strong metal chelator, like EDTA, added to the sample could improve tetracyclines recoveries. Tetracyclines have a strong tendency to form chelates with metal cations that may be present in the sample and EDTA can replace tetracyclines in those chelates (Reverté et al. 2003). However, no significant improvement in SPE recovery rates was observed when EDTA was added to the samples, probably because at an acidic pH, like that selected to this method, the formation of these chelates is not expected (Figure 13). Therefore, in the work conditions, the use of a chelator for recovery improvement was discharged.

The SPE extraction recoveries were determined for all of the different WWTP effluents, previously acidified at pH 2.0 and doped with five drugs at two concentration levels per drug: 40 and 200 $\mu\text{g L}^{-1}$. Recovery values reported in Table 4 are average values obtained for the

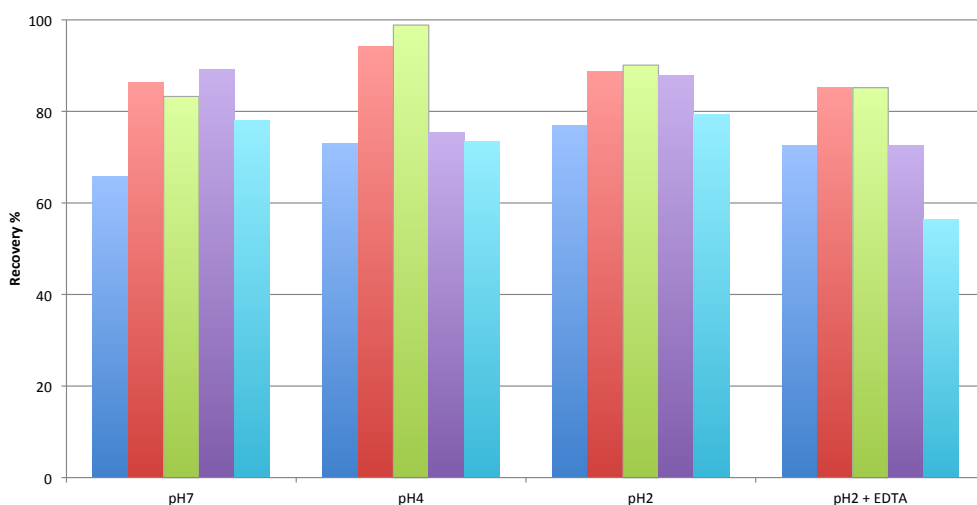


Figure 13 Solid-phase extraction (SPE) recoveries (%) observed with different sample pH (and EDTA addition for pH 2) for each drug (■minocycline (MNC); ■oxytetracycline (OTC); ■tetracycline (TET); ■enrofloxacin (ENR); ■ceftiofur (CEF)).

eight different wastewater samples. Despite the fact that samples have different characteristics in accordance with their origin (urban, slaughterhouse or pig farm), recovery values were similar with average recovery values presenting, in general, RSD lower than 20 %, indicating that matrix effects due to the different characteristics of the diverse wastewaters selected were minimal. Recovery values were also similar at both concentration levels, being in most cases higher than 80 %. The obtained results of SPE extraction recoveries for the tetracycline family (TET, OTC and MNC) and for the fluoroquinolone ENR are in agreement not only to previous published data for swine and slaughterhouse wastewater effluents (Ben et al. 2008, Shao et al. 2009, Tagiri-Endo et al. 2009, Tong et al. 2009) but also for other type of samples as wastewater from pharmaceutical industry (Asperger et al. 2009, Babić et al. 2006), urban WWTPs (Yang et al. 2005), or even surface water (Choi et al. 2007).

The overall precision of the method was determined based on the variation of the recovery yields obtained at the two different levels ($40 \mu\text{g L}^{-1}$ and $200 \mu\text{g L}^{-1}$) for all the different doped WWTP effluents samples. Relative standard deviations varied between 10 and 14% (Table 4).

The LOD and LOQ, initially determined for the HPLC method based on the signal-to-noise ratio (S/N) of 3 and 10, respectively, were recalculated considering the volume of real wastewater sample subjected to SPE pre-concentration (50 mL), being further on confirmed by analyzing decreasing concentrations of doped WWTP effluents until the signal was lost. Results obtained for LOD ranged from 0.8 to $3 \mu\text{g L}^{-1}$ whereas for LOQ values ranged from 2 to $10 \mu\text{g L}^{-1}$ (Table 4).

Table 4 Solid-phase extraction (SPE) followed by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) method characteristics determined, for the pharmaceutical minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF), in wastewater effluent samples. Values presented are average values obtained for 8 different wastewater samples

Pharmaceutical	Recoveries (%)		LOD ^a ($\mu\text{g L}^{-1}$)	LOQ ^b ($\mu\text{g L}^{-1}$)	RSD ^c (%)
	Level 1	Level 2			
	40 $\mu\text{g L}^{-1}$	200 $\mu\text{g L}^{-1}$			
MNC	76 \pm 18	68 \pm 12	2	6	10
OTC	89 \pm 18	87 \pm 7	2	6	10
TET	90 \pm 12	86 \pm 5	2	6	11
ENR	83 \pm 11	87 \pm 6	0.8	2	10
CEF	68 \pm 17	80 \pm 8	3	10	14

^{a,b} Limit of detection (LOD) and limit of quantification (LOQ) were initially determined based on the signal-to-noise ratio (S/N) of 3 and 10, respectively, and confirmed by analyzing decreasing concentrations of doped samples until the signal was lost. Volume of sample subjected to SPE: 50 mL.

^c Relative standard deviation determined based on the variation of the recovery yields obtained at the two different levels (40 $\mu\text{g L}^{-1}$ and 200 $\mu\text{g L}^{-1}$) for the different doped samples.

The LOD and LOQ depend on the volume of the SPE extracted sample, which in turn depends on the complexity of water matrix. In the present work, a relatively low volume (50 mL) of real wastewater sample was chosen to prevent the clogging of the SPE cartridges, however in most cases an higher volume (up to 250 mL) could be used which would improved the LOD and LOQ values. The LOD and LOQ values obtained in the present work were of the same order of magnitude than those found for some of the tetracyclines and fluoroquinolone determined by SPE-HPLC-DAD in a different effluent type (Buchberger 2007, De Zan et al. 2008) and similar to those obtained for swine wastewater by SPE-HPLC with UV detection (Malintan and Mohd 2006) for other veterinary antibiotics (sulfonamides). Despite the fact that the present LODs were higher, as expected, than those reported for more sensitive techniques, LC-MS or LC-MS/MS (Ben et al. 2008, Shao et al. 2009, Tagiri-Endo et al. 2009, Tong et al. 2009), the method here described is simple and with low matrix interference, allowing the identification of environmentally problematic wastewater effluents using equipment that is easily accessed in most laboratories.

Illustrative chromatograms of the pharmaceuticals studied in non-doped and doped effluent of WWTP 1 (with a concentration of 40 $\mu\text{g L}^{-1}$ and 200 $\mu\text{g L}^{-1}$ per drug) are shown in Figure 14.

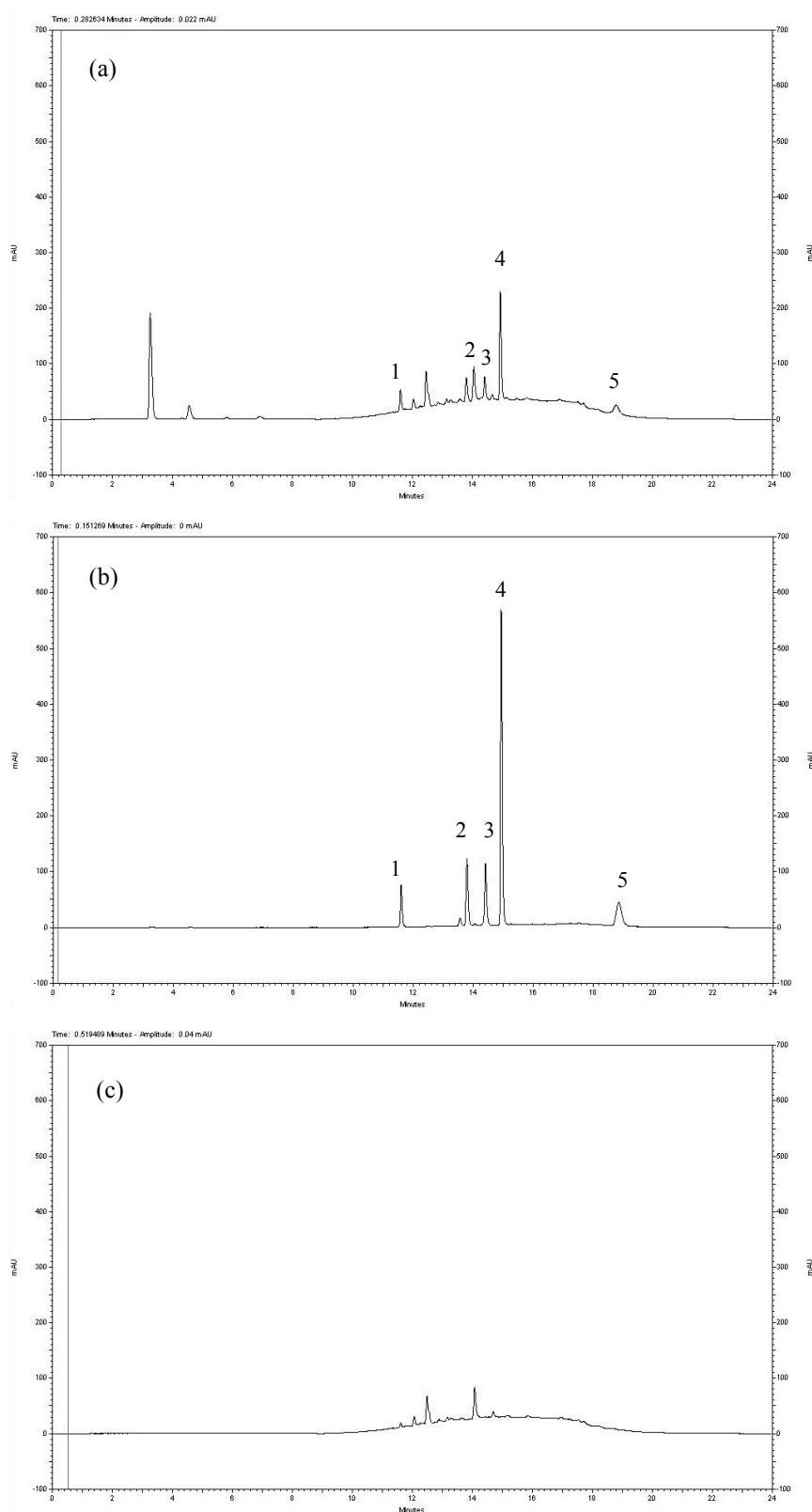


Figure 14 Typical chromatograms obtained by solid-phase extraction (SPE) followed by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) methodology of a wastewater effluent sample (a) doped at concentration level of $40 \mu\text{g L}^{-1}$, (b) doped at concentration level of $200 \mu\text{g L}^{-1}$ and (c) wastewater effluent (not doped). Peak numbers refer to: 1) minocycline; 2) oxytetracycline; 3) tetracycline; 4) enrofloxacin; 5) ceftiofur.

4.3.2 Survey of veterinary pharmaceuticals in WWTPs effluents

The previously optimized method was applied to the eight different WWTPs effluents (see section 2.3.1). Obtained results are shown in Table 5.

Enrofloxacin could be detected in one of the slaughterhouse WWTP effluents (SH WWTP 5) and TET could be quantified in the other slaughterhouse effluent (SH WWTP 6). MNC, OTC, TET and ENR could be detected and/or quantified in three urban WWTPs effluents (URB WWTP 1, 2 and 4) (Table 5). In the two pig farms WWTP effluents and one urban WWTP effluent (URB WWTP 3) drugs were not detected (< LOD).

The residual concentrations of drugs in livestock wastewater effluents can be used as indicators of veterinary drug use at such venues. Tetracycline family antibiotics can be both applied for human and veterinary purposes. Levels found in urban WWTPs effluents may be attributed to both, once it is known that several livestock industry effluents are discharged to the urban network before discharge into the environment. In fact, tetracycline was observed in an urban effluent that received a slaughterhouse effluent in which this compound was also

Table 5 Levels^a ($\mu\text{g L}^{-1}$) of pharmaceuticals, minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF), found in different urban (URB), slaughterhouse (SH) and livestock (LS) wastewater treatment plant (WWTP) effluents.

Pharmaceutical	MNC	OTC	TET	ENR	CEF
URB WWTP1	< 6 ^b	< 2 ^c	< 2 ^c	< 0.8 ^c	< 3 ^c
URB WWTP 2	< 2 ^c	7 ± 3	< 2 ^c	< 2 ^b	< 3 ^c
URB WWTP 3	< 2 ^c	< 2 ^c	< 2 ^c	< 0.8 ^c	< 3 ^c
URB WWTP 4	6.0 ± 0.5	< 2 ^c	6 ± 2	< 0.8 ^c	< 3 ^c
SH WWTP 5	< 2 ^c	< 2 ^c	< 2 ^c	< 2 ^b	< 3 ^c
SH WWTP 6	< 2 ^c	< 2 ^c	15.1 ± 0.6	< 0.8 ^c	< 3 ^c
URB WWTP	^d	^d	^d	0.0007 – 0.008 ^e	^d
Available literature				0.05 – 0.1 ^f	
SH WWTP	^d	0.01 – 3 ^g	0.01 – 1 ^g	0.002 – 0.4 ^g	^d
Available literature					
LS WWTP	^d	6 – 25 ^h	1 – 5 ^h	0.05 – 0.6 ⁱ	^d
Available literature		0.2 – 1 ⁱ	0.4 – 10 ⁱ		
		0.3 – 160 ^j			

^a Results presented as mean and standard deviation (n=3).

^b Limit of quantification

^c Limit of detection

^d Levels were not available in the literature

Range of concentrations reported by Jia et al. (2012)^e, Tong et al. (2011)^f, Shao et al. (2009)^g, Ben et al. (2008)^h, Tong et al. (2009)ⁱ, Tagiri-Endo et al. (2009)^j

detected. This could also be the reason why ENR, a drug of exclusive veterinary application, was detected in an urban WWTP effluent.

The detected veterinary drugs, OTC ($\leq 7 \mu\text{g L}^{-1}$), TET ($\leq 15 \mu\text{g L}^{-1}$) and ENR ($< 2 \mu\text{g L}^{-1}$), have also been reported in previous studies. In general, observed levels were higher than those reported for lake and ground water close to swine farms ($2 - 12 \text{ ng L}^{-1}$) (Tong et al. 2009) and for surface water receiving urban WWTP discharges ($10 - 19 \text{ ng L}^{-1}$) (Tong et al. 2011) and of similar order of magnitude to the levels reported for swine wastewater ($0.05 - 10 \mu\text{g L}^{-1}$) (Tong et al. 2009). However, levels of OTC reported by Ben et al. (2008) ($6 - 25 \mu\text{g L}^{-1}$) and by Tagiri-Endo et al. (2009) ($0.28 - 160 \mu\text{g L}^{-1}$) for swine wastewater tend to be higher than those observed in this work. In the analyzed livestock effluents no detectable levels ($< 2 \mu\text{g L}^{-1}$) of the drug were obtained. In contrast, the level observed for TET in slaughterhouse wastewater was higher than those previously reported ($0.01 - 1 \mu\text{g L}^{-1}$) (Shao et al. 2009).

Relatively to urban wastewaters, levels of ENR observed in this work were higher than those available in the literature ($0.7 - 108 \text{ ng L}^{-1}$) (Jia et al. 2012, Tong et al. 2011).

Veterinary drugs, namely ENR, TET, MNC and OTC, were detected for the first time in Portuguese urban, livestock and slaughterhouse wastewater effluents, through an expeditious method that allows screening for problematic levels of drugs in WWTP effluents.

The presence of these drugs is not routinely monitored and there is no regulation of their levels in water primarily because the knowledge of the input, fate and effect of most pharmaceuticals in the environment is limited. It is still not clear what these veterinary drugs and concentrations may mean or potentially can mean for the environment, i.e. for biodiversity of aquatic organisms, ecosystems functions and services. In fact, the modes of action of most pharmaceuticals in the environment are often poorly understood. In addition, the possible synergistic effects produced by the mixing of drugs are even more unknown. Data on chronic ecotoxicity are scarce and the scientific community understanding of potential ecotoxic effects mediated by pharmacological mechanisms of action is poor (Carlsson et al. 2006). Therefore, more ecotoxicological studies are needed, namely studies that merge the current bottom-up approach of ecotoxicology, that implies the use of small-scale experiments to predict effects on the entire ecosystems and landscapes, with a top-down macroecological approach directly focused on ecological effects at large spatial scales that considers ecological systems as integral entities as suggested by (Beketov and Liess 2012)

However, there are already indications that some pharmaceuticals are indeed problematic as shown by the inclusion of diclofenac in the list of priority substances under the European Water Framework Directive (WFD) or erythromycin in the contaminant candidate list (CCL 3) of United States Environmental Protection Agency (U.S. EPA).

Detection of some of the compounds more extensively used for therapeutic and prophylactic in Portuguese livestock industry indicates that measures should be taken to remediate this potential problem. Further research is needed not only to prevent the release of veterinary drugs to the environment as to study their environmental effects.

4.4 Conclusions

An expeditious analytical method for simultaneous analysis of five pharmaceuticals of three different families was applied to samples of different WWTPs effluents with probability of containing veterinary drugs.

Despite the fact that LODs are higher than those obtained by other techniques commonly applied to these determinations, this is an inexpensive and expeditious analytical technique that can be useful for signaling problematic wastewaters effluents.

This study reports for the first time the presence of veterinary drugs in WWTPs effluents in Portugal. The detection of some of the analyzed drugs in some samples shows that these effluents can be an important source of these drugs in the environment and that cost-effective approaches should be found to reduce/eliminate veterinary drugs from wastewater effluents.

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Chapter 5 - Multi-residue method for the analysis of veterinary pharmaceuticals in solid samples

5.1 Introduction

5.2 Experimental layout

5.2.1 Samples extraction optimization

5.3 Results and discussion

5.3.1 Extraction optimization

5.3.2 Method characteristics

5.4 Conclusions

References

5.1 Introduction

Although the presence of pharmaceutical compounds in wastewater has been widely described in the last years, there is scarce information about their concentration levels in generated sewage sludge and in river sediments affected by wastewater discharges (Martín et al. 2010). To perform flow studies and total mass balances in wastewater treatment plants (Ternes et al., 2005) and to study drugs effects in the environment, it is necessary the determination of concentration levels in the different stages of sewage sludge and in the soil/sediments affected by wastewater discharge.

However, analysis of pharmaceutical in solid matrices can be quite challenging. In fact, due to the often strong interactions between the drug residues and the solid matrices, the compounds are difficult to extract.

The analytical procedures usually comprise three steps, besides detection and data analysis: sampling, sample preparation for the extraction and separation of the compound through clean up of the extract as discussed in Chapter 1.

In this work a methodology for the simultaneous determination of MNC, OTC, TET (tetracycline family), ENR (a fluoroquinolone) and CEF (cephalosporin type) in sludge and sediment samples was pursued. An optimization of the drugs extraction using three of the most applied techniques (MAE, USE and VA) with different extraction solvents was applied independently for the different matrices. The final methodology characteristics including the extraction step, further sample cleanup and pre-concentration by SPE and analysis by HPLC-DAD were determined. This is the first time that MNC and CEF are determined in solid samples. For TET and OTC, as well as ENR, methodologies can be found in the literature although in family type approaches, only tetracyclines (Álvarez et al., 2010) or fluoroquinolones (Montesdeoca-Esponda et al., 2012; Sturini et al., 2010), not a simultaneous multi-family one.

5.2 Experimental layout

Equipment, material and reagents, as well as samples collection are detailed on Chapter 2. SPE and HPLC conditions used along this chapter are also described in Chapter 2.

Lyophilized solid samples were doped with a standard mixture to achieve a doping level of 20 $\mu\text{g g}^{-1}$ of each drug for sludge and 2 $\mu\text{g g}^{-1}$ for sediments. Method optimization and further characterization were carried out using these doped samples.

Sediment with higher fine fraction and higher organic matter content (grain size < 0.063 mm 15% and OM 8.4%), with expected stronger matrix effect, was used for method optimization (Sediment I). A different sediment sample (Sediment II) with less organic matter

and higher sand content (grain size < 0.063 mm 1% and OM 0.6%) were also analyzed for methodology reproducibility studies.

5.2.1 Samples extraction optimization

Solid samples extraction optimization was performed with six different solvents by three different extraction procedures, with fixed solvent volume and lyophilized sample mass (Table 6). The process of extraction was based on three commonly applied techniques: VA (5 min, Analog Vortex Mixer VWR (USA)), USE (15 min) and MAE (using a laboratory microwave system Ethos 1 Milestone (Sorisole, Italy)) with a temperature controlled procedure at 40 °C during 20 min). After extraction the slurry sample was centrifuged (2500 rpm for 5 min) and the supernatants further analyzed by HPLC, either with or without a SPE extraction.

Final optimized conditions are described on section 2.2.2.

Table 6 Solid samples extraction procedure optimization

Solvent (v:v)	Solvent volume	Sample mass	Process (extraction time)
Methanol/formic acid (96:4)			
Methanol/water (95:5)			
Methanol/acetone (95:5)	10 mL	0.5 g of sludge	Vortex agitation (VA) (5 min)
Methanol/HCl (1:1)		2 g of sediment	Ultrasonic extraction (USE) (15 min)
Methanol			Microwave assisted extraction (MAE) (20 min)
Acetonitrile/formic acid (99:1)			

5.3 Results and discussion

5.3.1 Extraction optimization

Solvents tested were chosen based on different literature applications for the extraction of pharmaceuticals (fluoroquinolones, tetracyclines and sulfonamides) from solid matrices (Arikan et al. 2008, Ferdig et al. 2005, Hu et al. 2010, Ok et al. 2011, Speltini et al. 2011). In addition, three of the most applied extraction techniques for pharmaceuticals analysis in solids

samples, VA, USE and MAE were compared in terms of efficiency and precision for each type of sample.

Sludge samples

Drugs recovery percentages were evaluated to attain the optimal extraction conditions for the studied pharmaceutical compounds in sludge samples (Figure 15). For VA none of the solvents could extract all the analytes. MAE and USE could extract all the analytes with the methanol/formic acid mixture, being recoveries obtained between 22 and 59% and 26 and 49%, respectively.

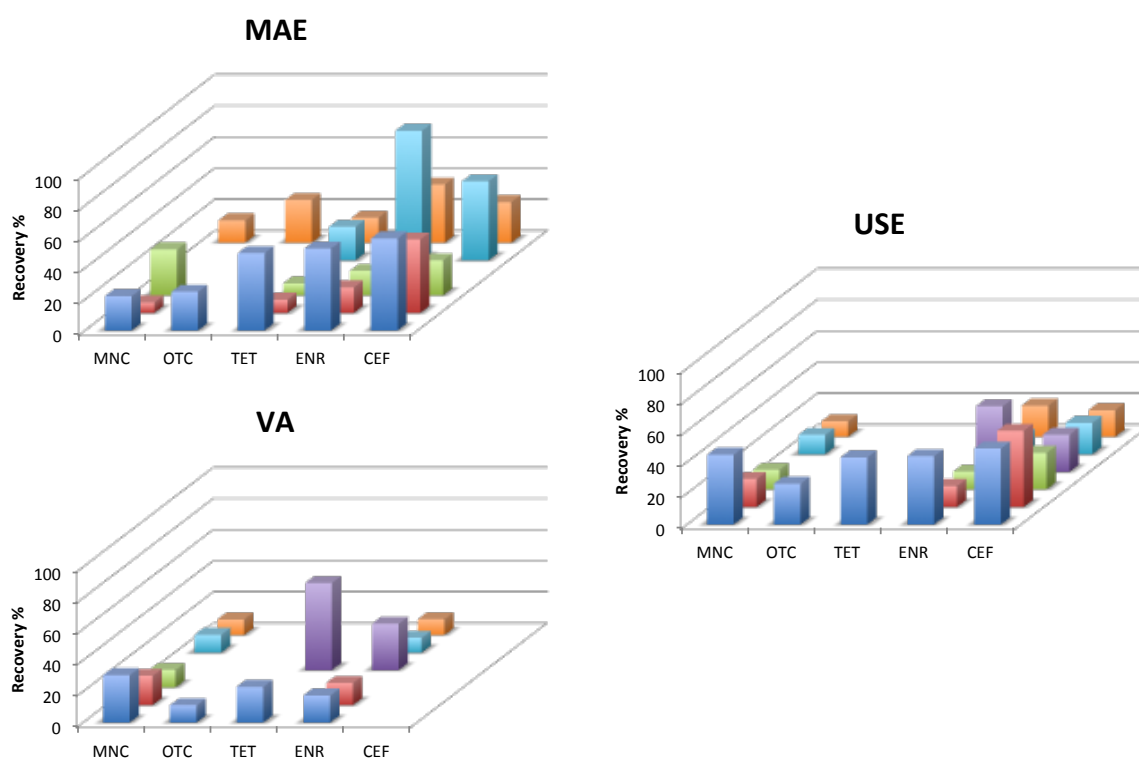


Figure 15 Drugs recoveries (%) of sludge samples doped at $20 \mu\text{g g}^{-1}$ for each drug, minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF) (mean values; $n=3$), observed using the different extraction techniques (microwave-assisted extraction (MAE), vortex agitation (VA) and ultrasonic solvent extraction (USE)) and the different solvents tested (■methanol/formic acid (96:4); ■methanol/water (95:5); ■methanol/acetone (95:5); ■methanol/HCl (1:1); ■methanol; ■acetonitrile/formic acid (99:1)).

Due to the low recovery efficiencies ($< 59\%$), further tests with sequential extractions were performed using the same solvent mixture and both USE and MAE to check the most suitable technique. Sludge was extracted with 10 mL methanol/formic acid (96:4, v/v) and, after centrifugation, the supernatant was collected. This procedure was repeated three times

and the supernatants combined to be further analyzed by HPLC. Results (Figure 16) showed a slight improvement for both techniques reaching recoveries between 31 and 79 %. The obtained results, although below the wide accepted analytical range of 80 to 120 %, were in agreement with those reported using methodologies available in the literature for different compounds and techniques for sludge samples (Chenxi et al. 2008, Hu et al. 2010, Jia et al. 2012, Kimura et al. 2007, Martín et al. 2010, Ternes et al. 2005). Both USE and MAE presented similar results for the target compounds, however USE afforded slightly higher recoveries (except for MNC) and lower standard deviation (n=6). In addition, simpler sample handling and lower extraction time led to the selection of USE as the best technique for the extraction of the selected multi-family veterinary drugs from sludge samples.

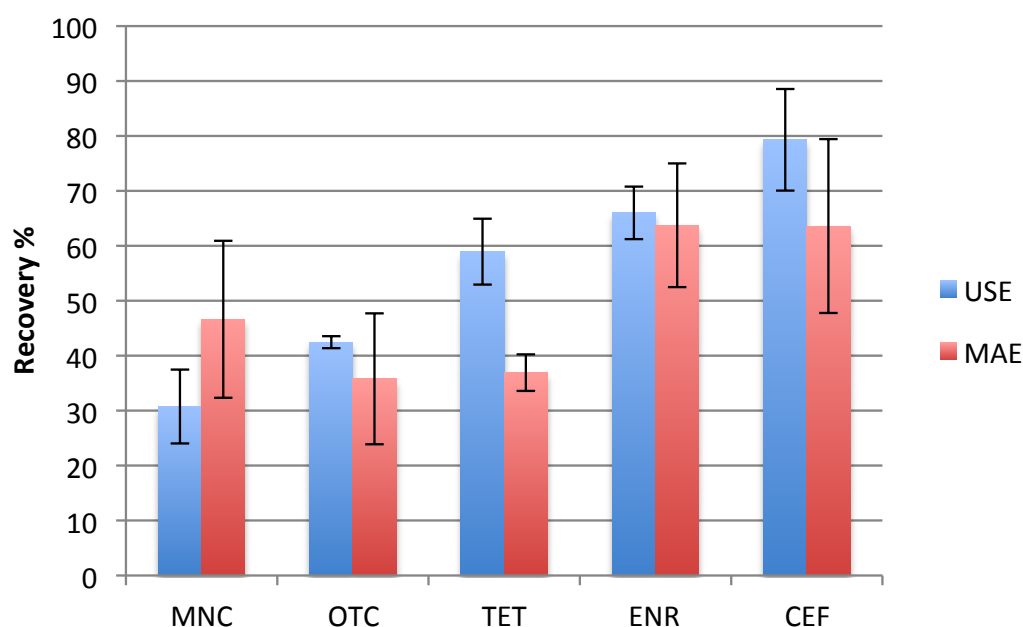


Figure 16 Drug recoveries (%) of sludge samples doped at $20 \mu\text{g g}^{-1}$ for each drug, minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF) (mean values; n=6), observed for the three sequential extractions with ultrasonic solvent extraction (USE) and microwave-assisted extraction (MAE) and using methanol/formic acid (96:4, v:v).

The procedure for sample cleanup and pre-concentration by SPE was evaluated in terms of recovery efficiencies once it could allow to improve methodology LODs. For SPE pre-concentration the homogenized extract (obtained in the previous selected condition by USE) was dissolved in water to attain an organic phase fraction lower than 5% and the final aqueous sample pH was adjusted to 2. The SPE procedure has been previously detailed in section 2.2.1. Extract samples (SPE A) were processed as described previously. To analyze the SPE cartridge retention capacity two sequential SPE extractions were performed. For that, the aqueous

samples passed through a first cartridge were collected and further processed again as a new sample through a new SPE cartridge (SPE B) (Figure 17).

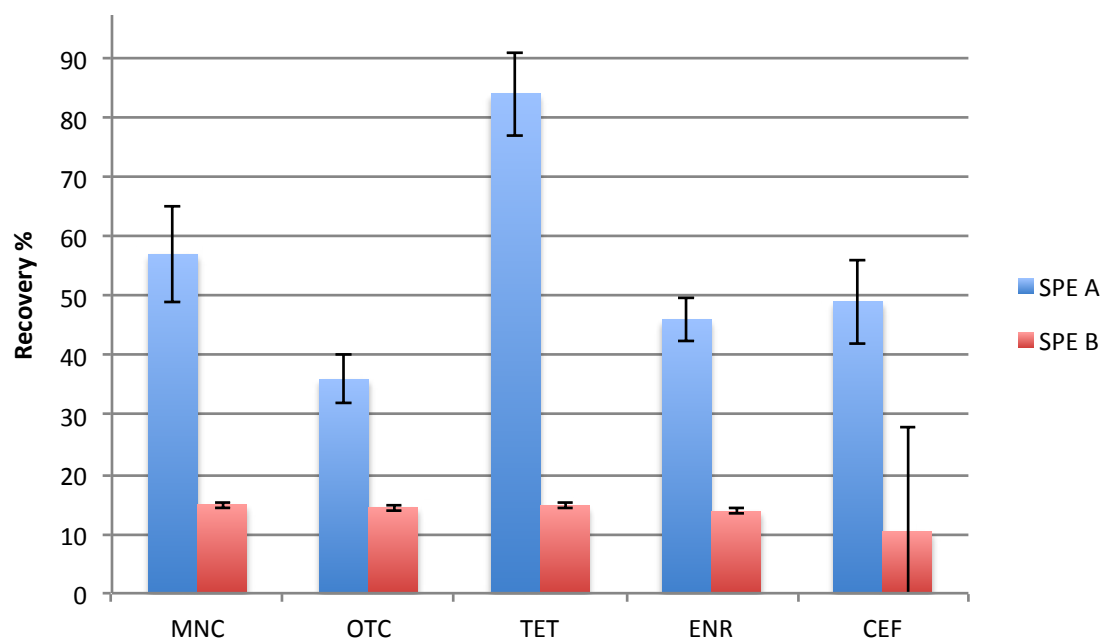


Figure 17 Solid-phase extraction (SPE) methodology recovery efficiency (mean values; $n=6$) tested with extracts obtained by ultrasonic solvent extraction (USE) with methanol/formic acid (96:4 v/v) of sludge samples doped at $20 \mu\text{g g}^{-1}$ for each drug, minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF) (two sequential solid-phase extractions (SPE) were performed SPE A and SPE B).

Results indicated that during the first SPE not all the target analytes were efficiently retained in the cartridge, probably due to the strong matrix complexity of sludge samples extracts. For wastewater samples SPE recoveries ranged from 76 to 90 % (Chapter 4). Present results (SPE A) showed lower overall methodology recoveries (ranging from 36 to 84 %), especially for OTC. However, recovery yields due to the extraction process (direct analysis) ranged already from 31 to 79 %. The recovered aqueous sample (SPE B) presented still about 15 % of the doping level. Nevertheless, SPE step introduces a ten times increase in methodology limits of detection.

Sediment samples

Drug recovery percentages obtained for sediment samples, using the different conditions of extraction (Table 6), were compared in order to reach the most promising conditions for multi-family veterinary drugs extraction. The mixture methanol/HCl and acetonitrile/formic acid used through the different techniques resulted in extracts with very strong matrix

interferences with negligible recovery valuables especially for tetracycline compounds family. In general, all the three techniques presented similar extraction efficiencies for the different solvents tested (Figure 18). CEF was the compound with better results (< 66%) in opposition to ENR (< 8%), while tetracyclines presented at most 27% of recovery. Most promising conditions obtained were MAE with methanol/formic acid and both VA and USE using either methanol or methanol/acetone.

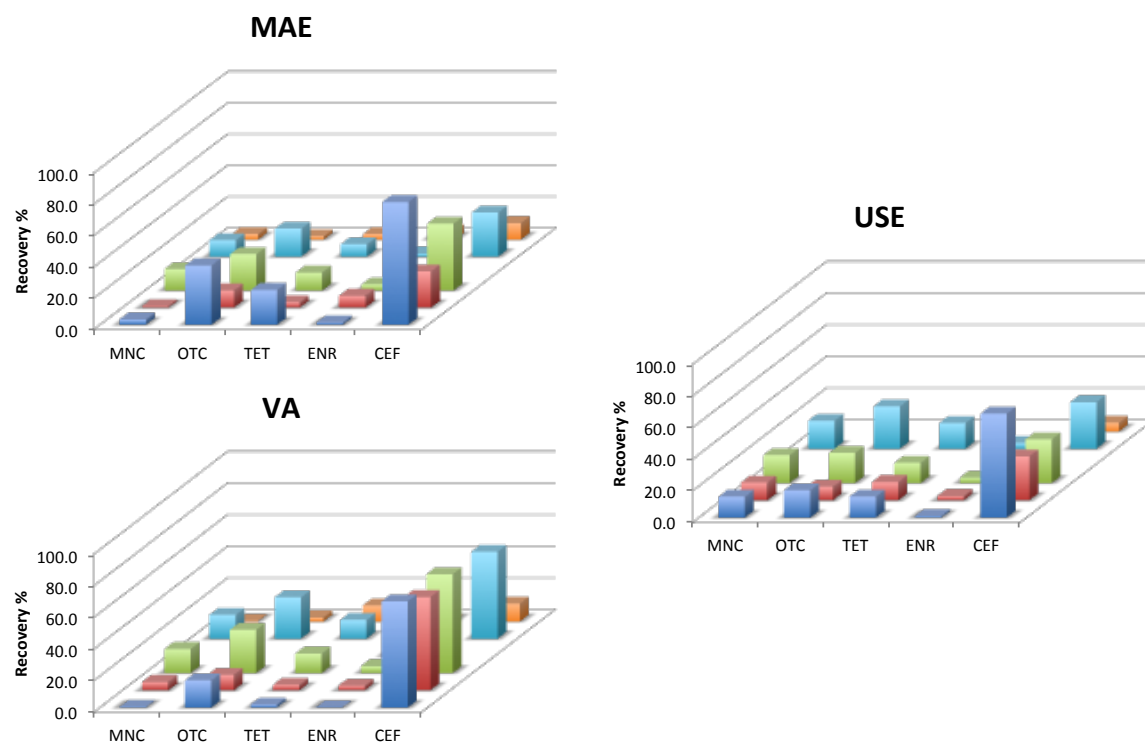


Figure 18 Drugs recoveries (%) of sediment samples doped at $2 \mu\text{g g}^{-1}$ for each drug, minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF) (mean values; $n=3$), observed using the different extraction techniques (microwave-assisted extraction (MAE), vortex agitation (VA) and ultrasonic solvent extraction (USE)) and the different solvents tested (■ methanol/formic acid (96:4); ■ methanol/water (95:5); ■ methanol/acetone (95:5); ■ methanol/HCl (1:1); ■ methanol; ■ acetonitrile/formic acid (99:1)).

However, due to poor recovery obtained for ENR further tests were conducted: 1) by increasing MAE program temperature to 80°C ; 2) using other solvents or mixtures of solvents and 3) using sequential extraction in USE.

MAE at 80°C was tested with all the solvents (Table 6) but results did not reveal any improvement in target compounds extraction. In fact, a secondary test with doped solvent mixture without sediment revealed the occurrence of compounds degradation, especially CEF, in all solvents (50 – 98% of loss; except acetonitrile/formic acid with only 3% loss). In addition, for all compounds extraction with methanol/formic acid and methanol/water mixtures resulted in 39 – 98% loss. These results clearly indicate that temperature is an important factor to be

controlled during the analytical procedure.

Solvents other than those in Table 6, namely hexane, dichloromethane, acetone and solvents mixtures, namely, acetone/formic acid (96:4 v/v) and acetone/methanol (50:50 v/v) were tested using USE, the less time consuming and easiest sample handling technique. With the exception of acetone/methanol (50:50 v/v) mixture, all extracts presented strong matrix interferences and poor recoveries. Results obtained with acetone/methanol (50:50 v/v) mixture were similar to those previously obtained with methanol containing mixtures.

Sequential extraction was then tested using USE for two and three cycle times of 15 min each for the initial solvents tested (Table 6). Comparing one and two sequential extractions (Figure 19), it was observed a recovery increase for ENR and a decrease for CEF. For tetracyclines the extraction percentages remained identical. The methanol/acetone mixture revealed the highest enhancement for ENR recovery (reaching 22%). The following third extraction did not increase further the recovery efficiency. In fact, for some solvents a decrease in efficiency was noticed, possible due to the higher sample manipulation needed in the process. Further tests with sequential extraction for VA and MAE were discarded once these techniques are more time consuming and, in addition for MAE, sample manipulation was already much higher than for the other two procedures.

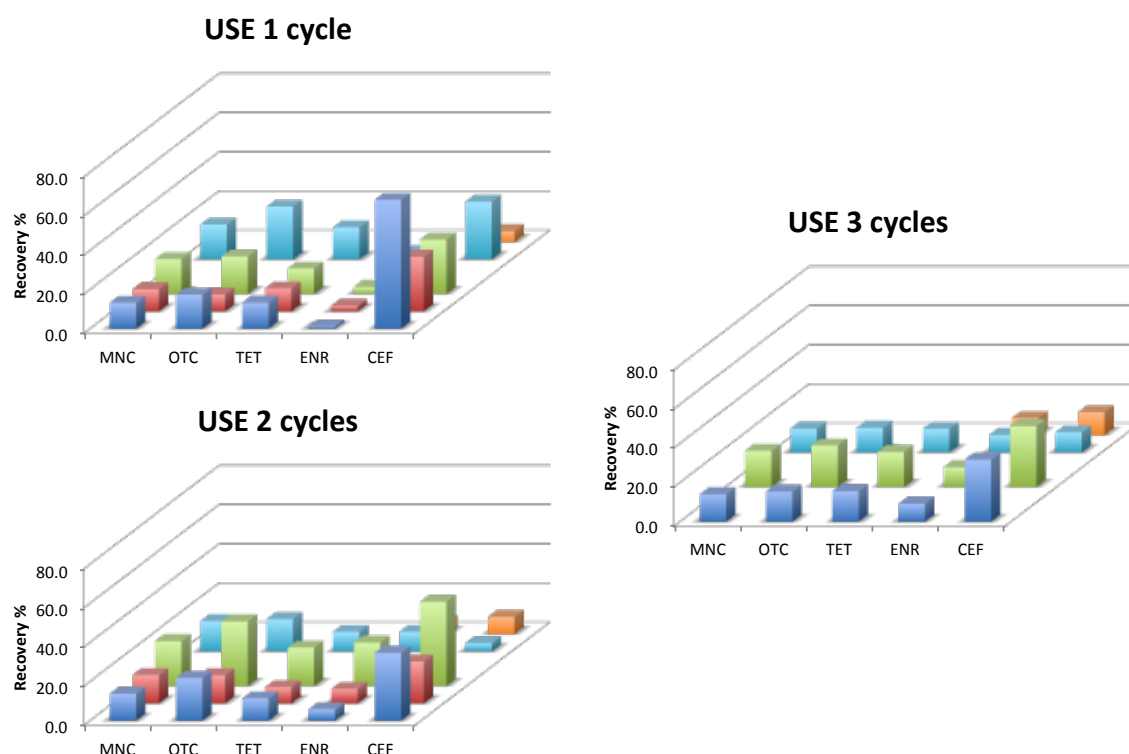


Figure 19 Drugs recoveries (%) of sediment samples doped at 2 µg g⁻¹ for each drug, minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF) (mean values; n=3), observed for one and the two and three sequential extractions using ultrasonic solvent extraction (USE) and the different solvents tested (■methanol/formic acid (96:4); ■methanol/water (95:5); ■methanol/acetone (95:5); ■methanol/HCl (1:1); ■methanol; ■acetonitrile/formic acid (99:1)).

Overall, results obtained presented a limited drug recovery from sediment matrix for all solvent/mixtures tested with the different techniques (VA, USE and MAE). Most promising results are detailed on Table 7. In general MAE by methanol/formic acid was better for OTC, TET and CEF extraction. VA provided similar recovery values but only for OTC and CEF by either methanol or methanol/acetone. USE sequential extraction provided an enhancement in ENR extraction resulting in a more balanced recovery value along the different compounds.

Table 7 Recoveries (%) of minocycline (MNC), oxytetracycline (OTC), tetracycline (TET), enrofloxacin (ENR) and ceftiofur (CEF) comparison for the most promising procedures with vortex agitation (VA), ultrasomic extraction (USE) and microwave assisted extraction (MAE), using direct and solid-phase extraction (SPE) processed sediment extracts

Procedure	Solvent	MNC	OTC	TET	ENR	CEF
Direct extract analysis						
MAE 40 °C	Methanol/formic acid	4	38	22	1	79
VA	Methanol/acetone	15	27	12	5	63
VA	Methanol	15	26	12	5	55
USE (2 cycles)	Methanol/formic acid	14	22	11	6	35
USE (2 cycles)	Methanol/acetone	26	32	23	22	43
SPE processed extract						
MAE 40 °C	Methanol/formic acid	11	14	11	9	16
VA	Methanol/acetone	9	12	10	9	11
VA	Methanol	10	19	15	11	16
USE (2 cycles)	Methanol/formic acid	11	12	9	8	23
USE (2 cycles)	Methanol/acetone	18	24	20	21	32

The usage of SPE was finally tested to research if matrix clean-up would enhance recoveries obtained. For that purpose, previously reported most promising techniques and solvents were tested by addition of a final step with SPE before HPLC-DAD determination. Therefore, for SPE pre-concentration the homogenized extract was dissolved in water to attain an organic phase fraction lower than 5%, the final aqueous sample pH was adjusted to 2 and the SPE procedure followed the description of section 2.2.1.

Results show a slight increase for some particular cases, however the most relevant fact is a broad reduction, possible due to the increase in sample manipulation and consequent losses (Table 7). Therefore, for the final methodology SPE was discarded.

The final methodology selected was the USE sequential extraction (2 cycles) by methanol/acetone with direct analysis of the extracts. This procedure allows the simultaneous analysis of six samples within 1 hour plus 2 hours for extract drying previously to the HPLC run.

5.2.2 Method characteristics

The methodology characteristics were determined for each type of matrix in terms of precision and limits of detection (LOD) and quantification (LOQ). These characteristics were determined for each type of extract used, direct or SPE processed. The HPLC-DAD methodology LOD and LOQ, determined based on the signal-to-noise ratio (S/N) of 3 and 10, respectively, and further confirmed by analysis of decreasing concentrations, (Chapter 4) were used to extrapolate the current overall methodology limits.

Sludge samples

For drugs analysis in sludge, limits were calculated considering the extraction of 0.5 g of sample. Values ranged from 0.8 to 3 $\mu\text{g g}^{-1}$ for LOD and from 2 to 10 $\mu\text{g g}^{-1}$ for LOQ for direct extract analysis (Table 8). The pre-concentration step of SPE allows lowering the limits 10 times from 0.08 to 0.3 $\mu\text{g g}^{-1}$ and from 0.2 to 1 $\mu\text{g g}^{-1}$ for LOD and LOQ, respectively. Current overall SPE-HPLC-DAD method limits are ca. ten times higher than those obtained for other pharmaceuticals by HPLC methodologies with fluorescence detection (LOQs of 0.003 – 0.4 $\mu\text{g g}^{-1}$ (Martín et al. 2010)) or with LC-MS, (0.009 $\mu\text{g g}^{-1}$ for ENR LOD (Jia et al. 2012), 0.004 – 0.015 $\mu\text{g g}^{-1}$ for TET and OTC LODs (Chenxi et al. 2008, Hu et al. 2010)).

The overall precision of the methodology was determined based on the extraction of 6 doped sludge samples, at a 20 $\mu\text{g g}^{-1}$ level, being the RSD lower than 6 % for direct analysis of the extracts and 14 % for SPE processed ones.

Table 8 Overall method characteristics determined using direct and solid-phase extraction (SPE) processed sludge extracts analyzed by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD)

Pharmaceutical	R^{2a} (n=8)	Direct extract analysis				SPE processed extract			
		LOD ^b ($\mu\text{g g}^{-1}$)	LOQ ^b ($\mu\text{g g}^{-1}$)	RSD ^c (%)	Recovery ^c (%)	LOD ^b ($\mu\text{g g}^{-1}$)	LOQ ^b ($\mu\text{g g}^{-1}$)	RSD ^c (%)	Recovery ^c (%)
Minocycline	0.993	2	6	6	31	0.2	0.6	14	57
Oxytetracycline	0.985	2	6	3	42	0.2	0.6	4	36
Tetracycline	0.995	2	6	3	59	0.2	0.6	7	84
Enrofloxacin	0.995	0.8	2	3	66	0.08	0.2	4	46
Ceftiofur	0.994	3	10	6	79	0.3	1	7	49

^a Correlation coefficient determined for the linear range (2 - 200 $\mu\text{g g}^{-1}$).

^b Limit of detection (LOD) and Limit of quantification (LOQ) determined based on HPLC methodology (Cavenati et al., 2012) extrapolated for the extraction of 0.5 g of sludge samples.

^c Overall precision and recovery of the methodology were determined at a 20 $\mu\text{g g}^{-1}$ doping level (n=6).

A possible matrix effect of the extract on HPLC drug quantification was tested. For this purpose, using both direct and SPE processed extract, a comparison of results obtained with standard addition method with those obtained using external calibration with aqueous mixed standards solutions was carried out. As statistically significant differences were not found, daily external calibration was further used, which has the advantage of being much less time consuming.

For the best-optimized conditions, recoveries, determined by analysis of real sludge samples (doped at a $20 \mu\text{g g}^{-1}$ level) ranged from 31% for MNC to 79% for CEF in the direct extract analysis and 36% to 84% (for the same compounds) in the SPE pre-concentrated extracts. The obtained results are in agreement with previous published data for a variety of other pharmaceuticals (37 – 78%) (Ternes et al. 2005) and also for TET (37 %) (Chenxi et al. 2008) and for ENR (86%) (Jia et al. 2012) when USE was applied for sludge samples.

Sediment samples

For sediments, limits were calculated considering the extraction of 2 g of sample. For recovery determination, two different types of sediments were used as described above (section 5.2). Values ranged from 0.09 to $0.2 \mu\text{g g}^{-1}$ for LOD and from 0.2 to $0.6 \mu\text{g g}^{-1}$ for LOQ for direct extract analysis (Table 9). Current overall methodology limits were higher or of the same order of magnitude than those obtained for other pharmaceuticals by HPLC methodologies with fluorescence detection (LOQ of $0.001 - 0.2 \mu\text{g g}^{-1}$ (Martín et al., 2010)) and higher than those obtained with LC-MS (0.4 ng g^{-1} for ENR LOD (Montesdeoca-Esponda et al., 2012) and $0.3 - 2 \text{ ng g}^{-1}$ for tetracyclines LOQ (Kim and Carlson, 2007)).

The overall precision of the methodology was determined based on the extraction of 6 doped sediment samples, at a $2 \mu\text{g g}^{-1}$ level for both Sediment I and II, being the relative standard deviations (RSD) lower than 20 %.

A possible matrix effect on HPLC drug quantification in the extract was tested. For this purpose a comparison of results obtained with standard addition method, with those obtained using external calibration with aqueous mixed standards solutions was carried out. As statistically significant differences were not found, daily external calibration was further used, which has the advantage of being much less time consuming.

Recoveries determined by analysis of real samples (doped at a $2 \mu\text{g g}^{-1}$ level), ranged from 22% for ENR (Sediment I) to 66% for ENR (Sediment II) in direct extract analysis. The variability observed in recoveries obtained for the two sediments analyzed denote the strong matrix interactions occurring. It is known that tetracyclines tend to form chelate complexes with metals ions and β -diketones and are strongly sorbed to soils. Fluoroquinolones are also known to sorb in particular to clay minerals via cation bridging. The obtained results are in agreement or lower than those previously published for a variety of other pharmaceuticals (< 15 – 103 %) (Martín et al., 2010) and for tetracyclines (< 30 - 82 %) (Kim and Carlson, 2007)

Table 9 Overall method characteristics determined using sediment extracts directly analyzed by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD)^a.

Pharmaceutical	R ^{2b} (n=8)	Direct extract analysis				
		LOD ^c	LOQ ^c	RSD ^d	Recovery ^c	Recovery ^c
		($\mu\text{g g}^{-1}$)	($\mu\text{g g}^{-1}$)	(%)	Sediment I (%)	Sediment II (%)
Minocycline	0.993	0.2	0.6	20	26 \pm 3	36 \pm 7
Oxytetracycline	0.985	0.2	0.6	19	32 \pm 4	52 \pm 10
Tetracycline	0.995	0.2	0.6	15	23 \pm 3	51 \pm 7
Enrofloxacin	0.995	0.09	0.2	10	22 \pm 1	66 \pm 6
Ceftiofur	0.994	0.2	0.6	13	43 \pm 6	27 \pm 2

^a Sediment with higher fine fraction and higher organic matter content was used for method optimization and characterization (Sediment I). A different sediment sample (Sediment II) with less organic matter and higher sand content was also analyzed for methodology recovery confirmation.

^b Correlation coefficient determined for the linear range (0.2 - 20 $\mu\text{g g}^{-1}$)

^c Limit of detection (LOD) and Limit of quantification (LOQ) determined based on HPLC methodology (Cavenati et al., 2012) extrapolated for the extraction of 2 g of sediment samples already corrected for the methodology recovery.

^d Overall precision and recovery of the methodology were determined at a 2 $\mu\text{g g}^{-1}$ doping level (n=6) for sediment I and II being selected the higher value obtained.

and for ENR (96%) (Montesdeoca-Esponda et al., 2012). Due to the variability observed within sediment matrix, future application of the methodology should be accompanied by recovery tests on the specific substrate to ensure a proper quality assurance and control.

5.4 Conclusions

An expeditious analytical method for simultaneous analysis of five veterinary pharmaceuticals of three different families in solid samples from WWTPs was optimized. For sludge samples the method yielded good selectivity and, taking in account the complexity of the matrix, acceptable recovery results, in accordance with available data in the literature for similar analytical processes. For sediment samples the method yielded lower recovery values, due to the complexity of the sediment matrices, although in accordance with available data in the literature for similar analytical processes.

These optimized methodologies can be applied to studies of the fate of veterinary drugs in activated sludge processes of WWTPs, constructed wetlands beds and WWTPs recipient water bodies taking in account that adequate corrections for recovery percentages may be necessary.

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Chapter 6 - Activated sludge systems removal efficiency of veterinary pharmaceuticals from slaughterhouse wastewater

6.1 Introduction

6.2 Experimental layout

6.2.1 Batch experiments with nutrient solution

6.2.2 Batch experiments with wastewater

6.3 Results and discussion

6.3.1 Nutrient solution experiments

6.3.2 Wastewater experiments

6.3.3 Soluble fraction approach and comparison with traditional WWTPs efficiency studies

6.4 Conclusions

Reference

This chapter is in submission process

6.1 Introduction

As discussed on Chapter 1, veterinary pharmaceuticals are widely used, which can result in a significant release of these pharmaceuticals into the environment.

Currently WWTPs are not designed to deal with pharmaceuticals. In fact, conventional WWTPs often only partially remove this type of compounds (Brown et al. 2006, Carballa et al. 2004, Clara et al. 2005, Miao et al. 2004, Ternes 1998). When xenobiotics enter a WWTP, they are not usually completely mineralized. They can be either partially retained in the sludge or metabolized to a more hydrophilic, but still persistent form. Therefore, they pass through the WWTP and end up in the receiving waters (Radjenovic et al. 2007). A major factor influencing pollutants removal from water is their ability to interact with solid particles, either natural or added to the medium (active carbon, flocculants/coagulants). Compounds with low adsorption coefficients tend to remain in the aqueous phase, which favors their mobility through the WWTPs (Carballa et al. 2004). On the other hand, compounds with high affinity for the sludge may be removed either by physical-chemical (settling, flotation) or biological processes (biodegradation). However, sorption to sludge may lead to an overestimation of the activated sludge treatment plants efficiency for drugs removal since it can occur the release of persistent molecules after biomass death (Prado et al. 2009b).

A growing number of studies worldwide have shown the presence of several different veterinary pharmaceuticals in surface and ground waters, river sediments and in soils at concentrations that could have potential impacts on the ecosystems (Zhao et al. 2010 and references therein) indicating that animal wastes, namely from livestock facilities or slaughterhouses, can be significant sources of these compounds despite their treatment. Livestock wastewaters, for instance, are usually treated through activated sludge processes and anaerobic or aerobic digestion processes (Prado et al. 2009a). However, knowledge on the removal processes of veterinary drugs from livestock and slaughterhouses wastewaters during its biological treatment is still very limited.

The object of this study was to evaluate the efficiency of a conventional wastewater treatment process (activated sludge) used for the treatment of wastewater containing ENR, CEF and TET.

To date, most studies on drugs removal in WWTPs focused on their occurrences and concentrations in influent, effluent and sludge. The removal efficiencies of the detected drugs were usually calculated on the basis of the field sampling results and vary greatly among different studies. To overcome the uncertainty in the investigation of the drugs removal routes, a few works have been conducted with activated sludge in controlled laboratory reactors at drugs environmental relevant concentrations (Li and Zhang 2010). In addition, there are already several studies on human pharmaceuticals removal from domestic wastewaters during conventional wastewater treatment process. But livestock and slaughterhouses wastewaters present normally higher organic loads that may affect efficiency along treatment, so studies

with this type of wastewater must be carried out.

Therefore, in this work a series of laboratory-controlled experiments in batch reactors were carried out on both (i) synthetic medium and (ii) real slaughterhouse wastewater.

6.2 Experimental layout

Material and reagents, samples collection of both sludge and wastewater, analytical methodologies and experiment set-up are detailed on Chapter 2.

Due to practical reasons batch reactors were just rinsed with soap and water and air-dried.

Once methodology used was developed for wastewater samples (Chapter 4) it was re-evaluated for nutrient solutions. Therefore, method recovery percentages were 86, 87 and 80 % for TET, ENR and CEF, respectively, and the overall variability of the method was below 14 %. The LODs in this work, due to a higher volume of sample used (250 mL) for both nutrient solution and wastewater were 0.4, 0.2 and 0.6 $\mu\text{g L}^{-1}$ for TET, ENR and CEF, respectively.

Sludge samples were analyzed accordingly the developed methodology (Chapter 5), being the results obtained corrected by the recovery percentage of the drug.

6.2.1 Batch experiments with nutrient solution

Nutrient solution was initially chosen as preliminary testing condition, once it allows to have an aquatic medium with essential nutrients and simultaneously be less prone to matrix interferences and less prone to influence drug bioavailability, such as expected for wastewater.

Drug concentration selected represent real concentrations previously found for veterinary drugs release into the environment, e.g. ENR (Babić et al. 2010) and oxytetracycline (Ben et al. 2008).

The experiments were carried out along 10 days to study the effect of different HRTs on the removal of the pharmaceuticals.

6.2.2 Batch experiments with wastewater

Pre-treated wastewater used presented pH of 7.76, COD of 150 mg L^{-1} and 320 mg L^{-1} of TSS of which 68.8% were VSS.

6.3 Results and discussion

6.3.1 Nutrient solution experiments

The drug ENR presented a distinct behavior between control (reactors without activated sludge) and sludge reactors (Figure 20).

In control reactors, ENR concentrations in solution decreased significantly (ca. 62%) during the 10 days experiment. This removal can be attributed to adsorption to reactors, photodegradation, volatilization and oxidation due to reactors aeration. In fact, based on the difference between the added and the real ENR concentration measured in the T_0 solution (right after ENR addition to the solution in the reactor), it can be concluded that 18% of the 62% ENR removal can be attributed to loss in the system due to drug adsorption to the batch reactors. In addition, previous published data showed that ENR can undergo direct photolysis a process that is conditioned by the presence of humic acids shielding effects (Schmitt-Kopplin et al. 1999). On the other hand, removal due to volatilization can be ignored on the basis of the high molecular weight as well as the presence of several polar groups in this antibiotic. However, microbial degradation can also be responsible for some drug removal. In fact, although a synthetic doped solution was used, it was not sterilized and the nutrients favored a microbial community increase in solution without drug addition along the 10 days period (TCC $5.59 \log_{10} \text{ mL}^{-1}$ at T_0 and $6.53 \log_{10} \text{ mL}^{-1}$ after 10 days). Due to technical reasons TCC could not be determined in ENR control solutions. But the presence of microbial communities in ENR control reactors cannot be ruled out and therefore microbial degradation could have contributed to the observed drug's removal from the aqueous phase.

In sludge reactor, ENR concentration in solution at T_0 showed a higher loss of the compound (68%) than in control reactors, indicating in this case also adsorption of the drug to the sludge, which was confirmed by the detection of ENR in the sludge (solid fraction). In fact, ENR concentrations measured in sludge at T_0 was around $7 \mu\text{g g}^{-1}$.

The concentration of ENR in solution at day 10 was of $31 \mu\text{g L}^{-1}$, which represented a total removal of 69% from the aqueous phase. Despite the fact that ENR concentrations at T_0 and after 10 days were similar, both in solution and sludge, they varied throughout the treatment. This fact may be related with variations in the partitions between aqueous (solution) and solid fractions (sludge). In addition, mass balance (Figure 21) clearly shows drug removal/degradation along time. In fact, ENR mass balance indicates that, after 10 days, 31% of added ENR was still in the aqueous phase, 14% was adsorbed to the sludge and only 55% of ENR was effectively removed/degraded during the treatment, either through adsorption to reactors, photodegradation, oxidation due to reactors aeration or microbial degradation by microorganisms present in the medium due to sludge addition. In sludge reactors a possible photodegradation may be much more reduced due to the observed turbidity (a consequence

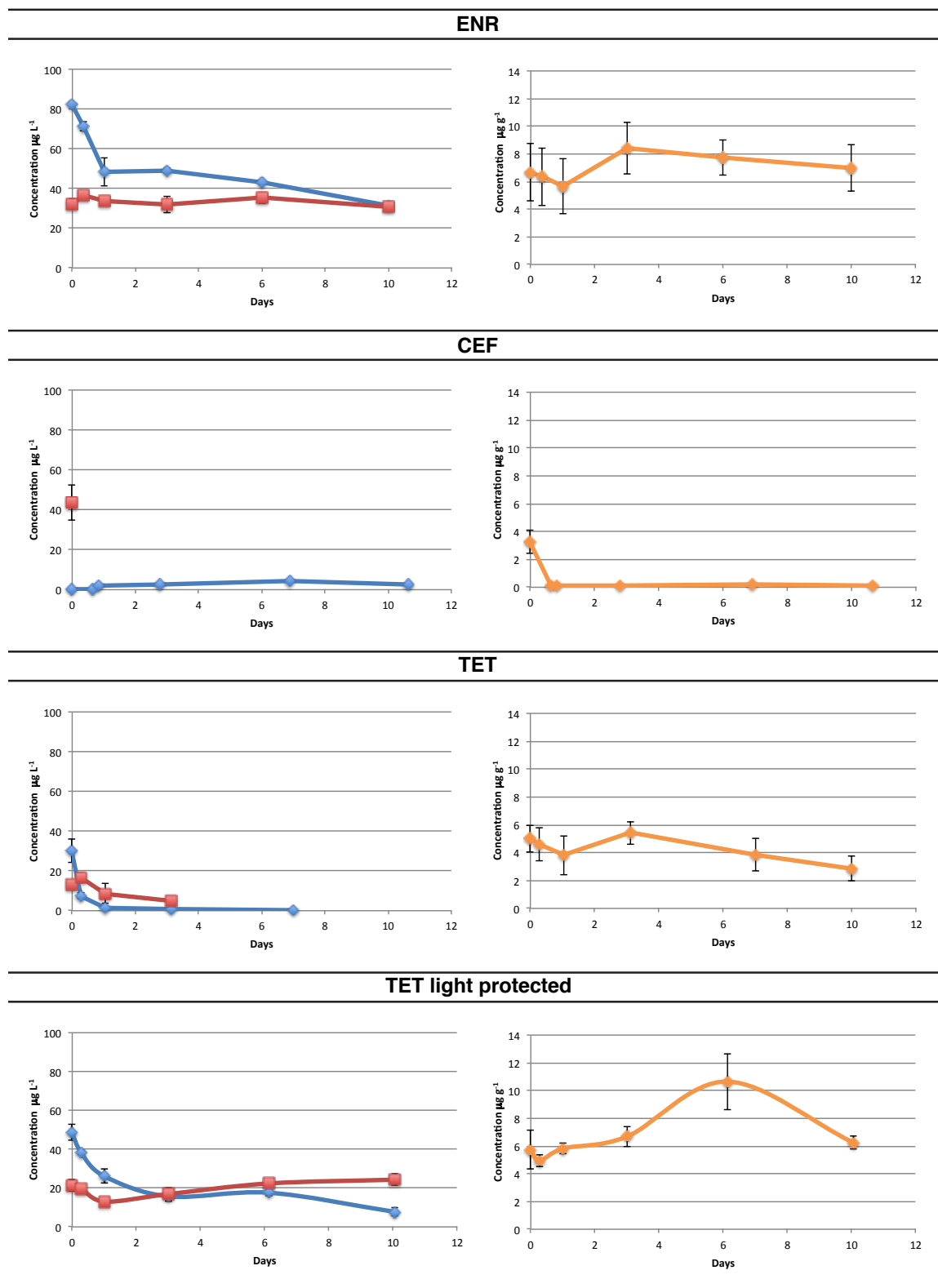


Figure 20 Drug, enrofloxacin (ENR), ceftiofur (CEF) and tetracycline (TET), concentrations measured in solution in ■ control reactor and ■ sludge reactor and in ■ solid sample (sludge), in the nutrient solution experiments.

of system agitation). Adsorption to reactors could also be lower than that observed in control reactors due to the presence of sludge that would also compete for the adsorption of the drug.

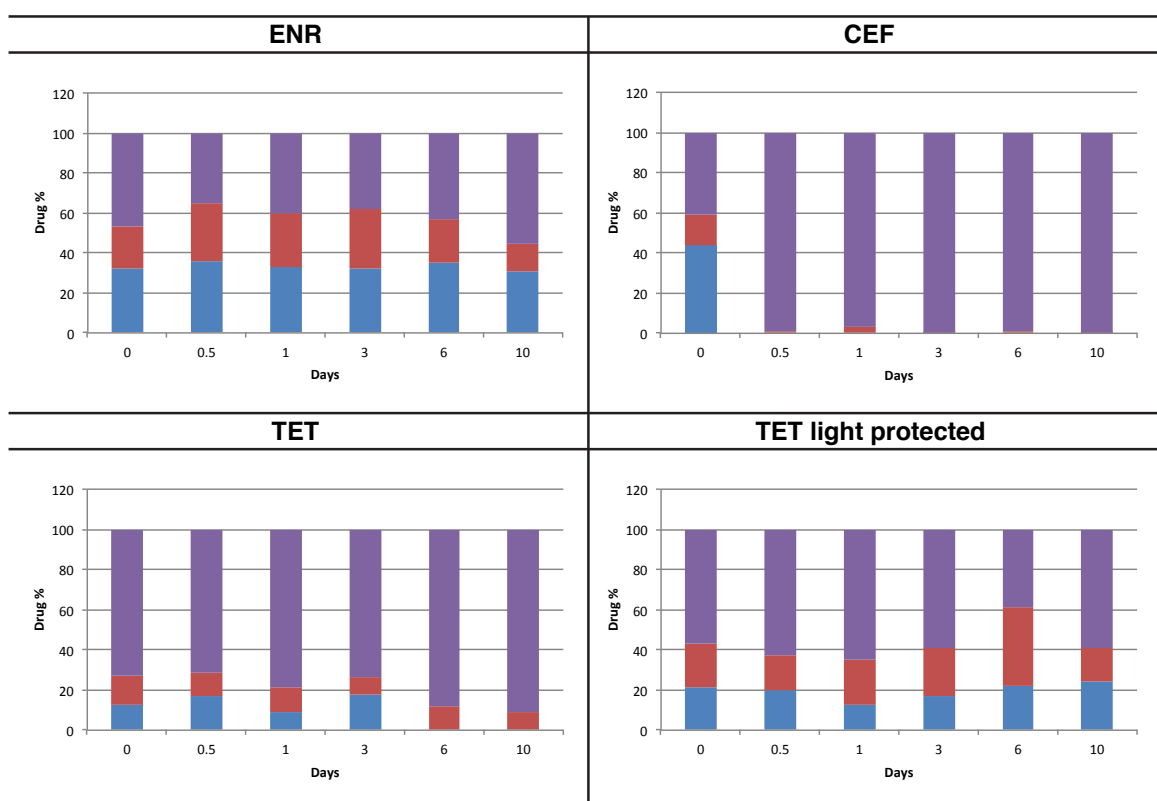


Figure 21 Mass balance observed for the drugs enrofloxacin (ENR), ceftiofur (CEF) and tetracycline (TET) in sludge reactors in the nutrient solution experiments: ■ drug in solution, ■ drug in sludge and ■ drug removed.

The control reactors in CEF assay revealed that only a residual level of the compound could be detected in solution during the 10 days of the experiments (Figure 20). There was a significant removal of the drug from the solution by adsorption to the system even at T_0 . In sludge reactor, at T_0 , there was a loss of 56% of CEF amount from solution. The removal increased in a short time, being CEF not detected after half a day. Apparently CEF was strongly bound to both the reactors and the sludge, being rapidly removed from the aqueous phase, as indicated by CEF mass balance (Figure 21). In fact, CEF concentrations in sludge were about $3 \mu\text{g g}^{-1}$ at T_0 , decreasing rapidly and being only slightly above the detection limit during the 10 days period. Although some microbial degradation may have occurred, significant adsorption of CEF to the reactors walls and tubing did not allow an efficient study of the fate of this drug in the activated sludge system.

Two separate assays were performed for TET, one without and another with light protection to prevent the known TET photodegradation (Werner et al. 2006). Tetracyclines, as a class, are highly photoreactive. In fact, several studies have shown that both TET and oxytetracycline are rapidly photodegraded, although sorption may dampen the photodegradation of these compounds in natural systems (Boreen et al. 2003).

Two distinct behaviors could be observed (Figure 20). Comparatively to reactors with light protection, reactors not protected presented much lower TET levels in solution, being TET undetectable after day 3 in sludge reactors and after day 6 in control reactors. On the other hand, when the reactors were light protected, TET was quantified in both control and sludge reactors solution along the 10-day period. TET could be quantified in sludge samples of both assays, being the concentration relatively higher in light protected reactors. These results point to a significant photodegradation of the drug with a significant removal from the solution as shown also by the mass balance (Figure 21).

In light protected TET assay, the TET loss from the solution at T_0 was 58% for control and 82% for sludge reactor. TET levels decreased continuously in control reactor solution to a final concentration of $7 \mu\text{g L}^{-1}$ (93% total removal of the added concentration), whereas in sludge reactor TET level in solution remained constant, around $20 \mu\text{g L}^{-1}$. This steady value along the 10 days of the experiment present a similar trend to that observed for ENR. TET decrease in control reactor solution can be attributed to adsorption and oxidation once photodegradation was controlled. Microbial degradation could have also contributed for this removal, although its contribution is unknown. In fact, although nutrient solution without any drug presented an increase in the microbial community along the 10 days period (as discussed above), TET control reactor solution presented significantly lower TCC ($< 5.29 \log_{10} \text{ mL}^{-1}$ at T_0 and $5.64 \log_{10} \text{ mL}^{-1}$ at 10th day, respectively), revealing the drug toxic effects. Therefore, microbial degradation cannot be excluded but may have been reduced due to TET toxicity.

TET sludge concentrations presented a high variation ranging from 4.9 to $10.6 \mu\text{g g}^{-1}$. However, this variation was not correlated with time or aqueous phase TET concentrations, being probably due to changes in the partitions between the solid and liquid phases. In sludge light protected reactor, TET mass balance (Figure 21) indicates that after the 10 days period 24 % of added TET was still in the aqueous phase, 17% of the mass was adsorbed to the sludge and 59% of TET was effectively removed/degraded during the treatment by adsorption to reactors and by microbial degradation.

The pH and VSS were identical among solutions with and without drugs and among different drugs. Solutions pH (6.5 at T_0) varied on the first day but then remained constant in control (around pH 7) and in sludge reactors (around pH 5). VSS were around 2 g L^{-1} in sludge reactors at the beginning of the experiment, decreasing slowly throughout the 10-day period to half. Therefore, for the nutrient solution experiment pH and VSS parameters were not related with the observed variability in drugs concentrations.

6.3.2 Wastewater experiments

The results obtained from the nutrient solutions experiments led to the preparation of a similar experiment where veterinary drugs degradation and/or removal was further studied using a real slaughterhouse wastewater for better understanding the behavior of real systems. CEF was not included in this study once the results with nutrient solution were inconclusive.

Enrofloxacin concentrations in solution (Figure 22) presented distinct levels but an equal trend for control (just wastewater) and sludge reactors.

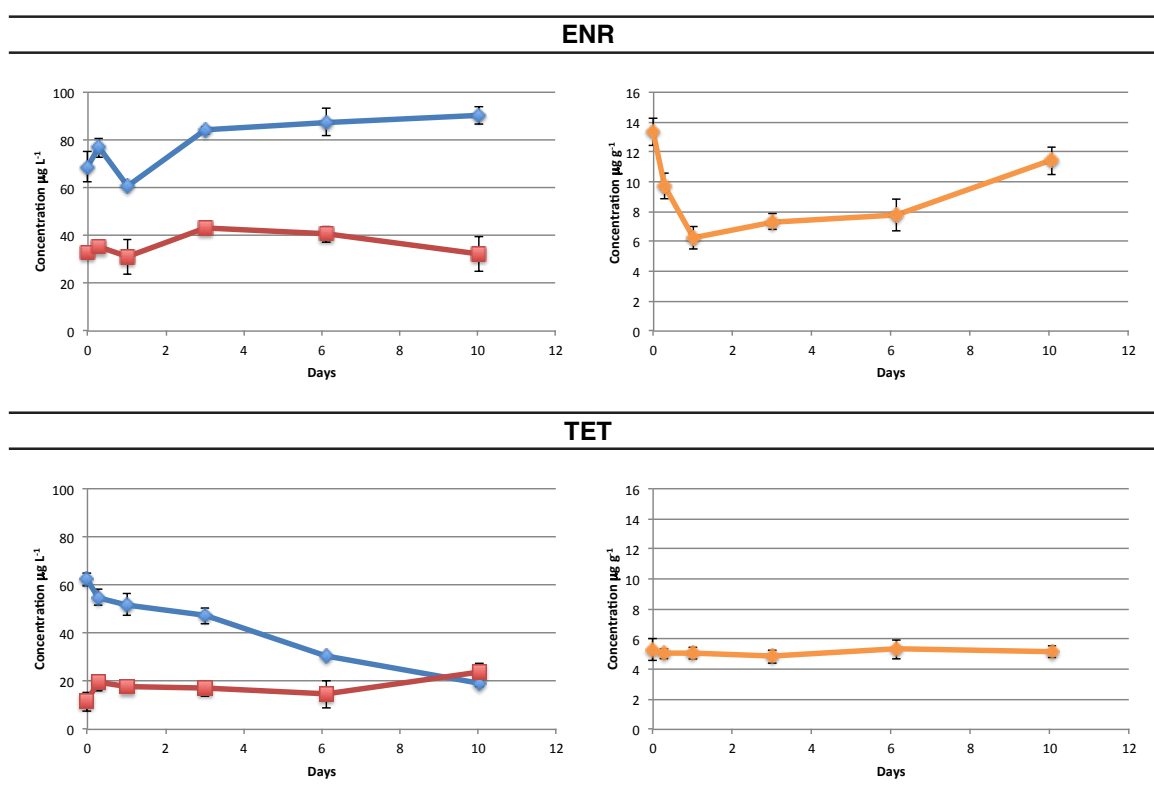


Figure 22 Drug, enrofloxacin (ENR) and tetracycline (TET), concentrations measured in solution in ■ control reactor and ■ sludge reactor and in ■ solid sample (sludge), in the wastewater experiments.

In control reactors there was a loss of 31% of added ENR at T_0 , but along the experiment ENR slowly increased its level in solution being of $90 \mu\text{g L}^{-1}$ at day 10. These values were higher than those observed in the nutrient solution experiment. In this case, the wastewater organic load promoted drug retention (by adsorption) in the aqueous phase and prevented some of the removal by adsorption to the system and by degradation. Microbial community decreased in wastewater without drug addition along the 10 days period (from TCC $6.49 \log_{10} \text{ mL}^{-1}$ at T_0 to

TCC $5.69 \log_{10} \text{ mL}^{-1}$) probably due to reduction of nutrients. ENR doped wastewater revealed even lower TCC values (from TCC $6.11 \log_{10} \text{ mL}^{-1}$ at T_0 to TCC $5.44 \log_{10} \text{ mL}^{-1}$) showing toxic effects due to the drug presence. Therefore, microbial degradation although expected may have been reduced due to ENR toxicity.

As before, solutions in sludge reactors presented higher ENR loss at T_0 (67%) than control ones, indicating again not only adsorption to reactors and to the wastewater organic load but also adsorption to sludge. ENR concentration in sludge reactor solution also remained constant along the 10-day period being $32 \mu\text{g L}^{-1}$ at day 10, a value similar to that observed in nutrient solution experiment. In addition, ENR concentrations in sludge varied between 4 and $14 \mu\text{g g}^{-1}$. Although these concentrations were relatively higher than those obtained previously for nutrient solution experiments they were not correlated with time or with solution levels. Therefore, the variations observed may be due to changes in the partitions between solution, wastewater organic matter and sludge.

Enrofloxacin mass balance (Figure 23) indicates that, after the 10 days period, 32% of added ENR remained in the aqueous phase, 33% of ENR mass was adsorbed to the sludge being only 35% of ENR effectively removed/degraded during the treatment. Although the overall removal from solution was similar to that observed in nutrient solution experiments, the ENR percentages adsorbed to the sludge were higher in the wastewater experiments. These data are in accordance with previous studies with quinolones, which indicated that they sorb significantly to soils and sludge (Golet et al. 2003, Yang et al. 2011).

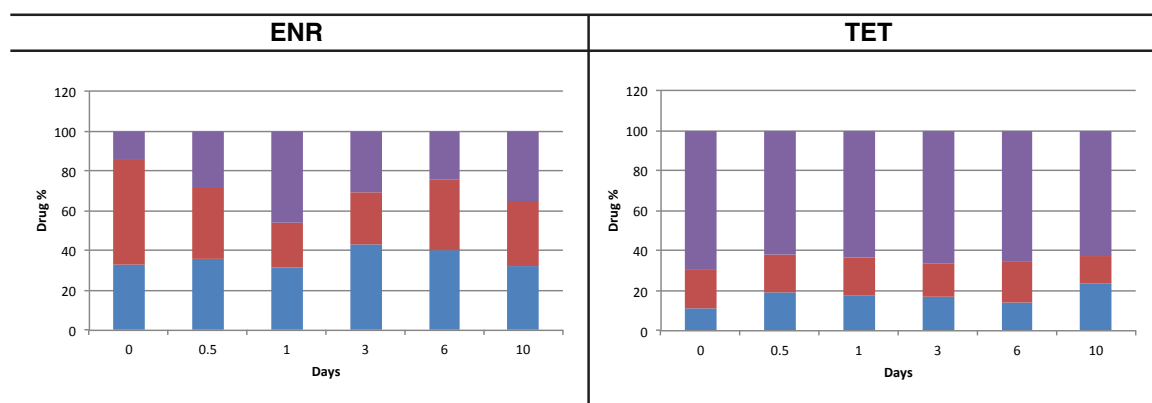


Figure 23 Mass balance observed for enrofloxacin (ENR) and tetracycline (TET) in sludge reactors in the wastewater experiments: ■ drug in solution, ■ drug in sludge and ■ drug removed.

Tetracycline study was performed with the reactors protected from light due to the important effect of photodegradation observed previously. In control reactor solution a loss of 38% of added TET at T_0 was observed. Along the experiment TET concentration decreased

until $19 \mu\text{g L}^{-1}$, representing 81% total removal of TET from the aqueous phase (Figure 22). Besides adsorption, this decrease may be attributed to oxidation and to microbial degradation (from the microbial community present in the wastewater). Microbial community in TET doped wastewater showed lower TCC values than those in non-doped wastewater (although results were not always significantly different). Doping may have affected microbial activity (TCC $6.28 \log_{10} \text{ mL}^{-1}$ at T_0 to TCC $5.80 \log_{10} \text{ mL}^{-1}$). Nevertheless, the removal values from solution observed in control reactor in this experiment were slightly lower than those observed in the nutrient solution experiment, indicating that the organic load present in the wastewater also interfered with the sorption of TET to reactor and/or with TET degradation, as observed for ENR.

Sludge reactor solutions presented higher loss at T_0 (89%) than control ones indicating again adsorption of TET to sludge. Sludge drug concentrations measured were very similar (around $5 \mu\text{g g}^{-1}$) along the 10 days. TET concentrations in sludge reactor solution tend to slightly increase along the 10-day period being $24 \mu\text{g L}^{-1}$ at day 10. This behavior is different from that observed in nutrient solution experiment indicating that also for this drug the organic fraction of the wastewater had a significant impact on system behavior. Wastewater organic load may also adsorb TET influencing the amount of drug in each phase, which results from different partitions among solution, sludge and wastewater organic matter. So wastewater organic load was very important, conditioning not only the soluble concentration of this antibiotic in the aqueous medium but also its partitioning with the sludge fraction and, consequently, its availability for degradation during the biological treatment.

The TET mass balance (Figure 23) indicates that of the overall 77% removal from the aqueous phase at day 10, 14% of the mass was adsorbed to the sludge being only 63% of TET effectively removed/degraded during the treatment after the 10 days period. When comparing the TET mass balance in wastewater with the one observed in the previous nutrient solution experiments, results were very similar.

Previous published work tended to prove that biosorption was the most favorable route for tetracyclines fate into a biological system. However, those results were obtained with washed activated sludge before sorption test and it was considered that the organic content of real environmental matrix may modify sorption capacities (Prado et al. 2009a). At the light of our results, real matrix influence was confirmed. However, in this case, biosorption although existent was not the major route for TET removal from wastewater. Livestock and slaughterhouses generally present wastewaters with much higher organic loads than other wastewaters, such as urban ones, a factor that was found to be determinant in drugs partitioning in the aqueous medium and therefore, a factor that affects treatments efficiency.

Tetracyclines have been shown to form relatively stable complexes with particulates and metal cations, demonstrating a capacity to be more abundant in the sludge component. Depending on aqueous pH, presence of cations and light exposure, tetracycline degradation products can be formed, many of which are highly soluble and appear to be the most stable form in receiving waters (Watkinson et al. 2007). Contrary to our TET wastewater experiment,

in a real system photodegradation will also occur once the reactors are open and there is always light penetration in a certain extension, which will increase drug degradation on the wastewater treatment facility.

The fate of antibiotics is closely linked to the operating conditions of the biological process. The introduction of an antibiotic in a biological system can alter the operating conditions. In fact tetracycline, generally, presented inhibitory capacity (Prado et al. 2009a). In the present study, a clear toxicity of the drug to the microbial community of the aqueous media was observed. Even if antibiotics entering a process at the usual environmental values may not disturb the system, the activated sludge process may be a factor for bacterial antibiotic resistance (Prado et al. 2010). Therefore, the question of antibiotics effect on sludge still has to be clearly identified to prevent any environmental contamination.

The pH and VSS were identical among wastewater with and without drugs and with ENR or TET. Variations observed were very similar to the ones of the nutrient study, although initially wastewater presented a higher pH (around pH 7.5). Overtime solutions pH varied on the first day but then remained constant in control (around pH 8) and in sludge (around pH 5) reactors. VSS variation showed a similar behavior to that observed for nutrient experiments. Therefore, also for wastewater experiment pH and VSS parameters were not related with the observed variability in drugs concentrations.

6.3.3 Soluble fraction approach and comparison with traditional WWTPs efficiency studies

To date the removal efficiencies of the detected drugs have been usually calculated on the basis of the field sampling results of influent, effluent and sludge (Gracia-Lor et al. 2012, Li and Zhang 2010, Tong et al. 2011). Traditionally, aqueous samples are filtered, being the analytical quantification directed to the soluble drug fractions (Joss et al. 2006, Nakada et al. 2006, Tong et al. 2011). This type of approach discards an important fraction of the drugs adsorbed to the organic matter and particulate matter. Recently, a few authors have alerted to this fact (Deo and Halden 2010a, b, Wille et al. 2010), but apparently no repercussions have still occurred on more recent studies. In addition, Lahti and Oikari (2011) presented a first study measuring pharmaceuticals in settleable particulate material, reaching levels between 6 to 1350 ng g⁻¹ (dry weight) for different compounds. These results highlight the fact that measuring only the dissolved fraction of pharmaceuticals in the WWTP effluent may underestimate the loading and risks to the aquatic environment. Present study although also working with the soluble fraction in the water phase was based on compound dosing and mass balances were calculated with the known added amount of contaminants. This fact allowed concluding that wastewater organic load could retain a portion of the added pharmaceutical. Therefore, more attention

needs to be devoted for livestock and slaughterhouses effluents where organic loads are higher and consequently veterinary drugs release to the environment can be more problematic.

If present results would have been discussed as traditionally only refereeing to soluble drug concentrations, calculated mass balances should have been completely different. For that purpose, an approach was attempted discharging the known dosed concentration, just considering the measured levels in the soluble fraction of the control reactor (without sludge) at the begging of the experiment (T_0 values). For example, for ENR wastewater study, results (Figure 24) would have shown a higher adsorption to sludge (48% vs the real 34%) and a higher overall removal efficiency (86% vs the real 69%). Besides, this type of approach may have led to the appearance of negative removal percentages, as reported previously (Carballa et al. 2004, Castiglioni et al. 2006, Gracia-Lor et al. 2012, Joss et al. 2005, Matsuo et al. 2011). In fact, the presence of pharmaceuticals adsorbed to the organic load fraction could be released along treatment, disguising the real treatment efficiency and processes.

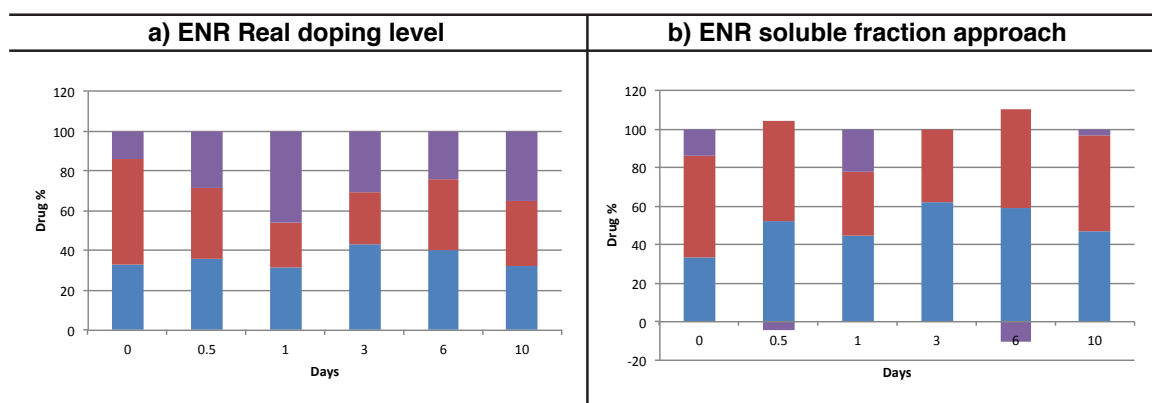


Figure 24 Mass balance observed in enrofloxacin (ENR) wastewater sludge reactors considering two methods of data analysis: a) real doping level, b) discarding particulate matter sorption (soluble fraction approach, level measured at the begging of the experiment ($69 \mu\text{g L}^{-1}$) in the control reactor (without sludge)) being ■ drug in solution, ■ drug in sludge and ■ drug removed.

It is known that compounds with low adsorption coefficients tend to remain in the aqueous phase, which favors their mobility through the WWTPs (Carballa et al. 2004), being more prone for degradation processes. On the other hand, compounds with high ability to interact with solid particles, both natural and added to the medium, besides removed by physical-chemical (settling, flotation) or biological processes (biodegradation), can also persist in the systems and further into the environment. In addition, sorption to sludge may lead to an overestimation of the activated sludge treatment plants efficiency for drugs removal since it can lead to the release of persistent molecules after biomass death (Prado et al. 2009b).

6.4 Conclusions

Results obtained in this study are consistent with the known fact that the major factor influencing the efficiency of pollutants removal from water is their ability to interact with solid particles. Present results indicate that sorption to sludge and, in a less extent, to the organic matter present in the wastewater were responsible for a significant percentage of drugs removal from wastewater during its biological treatment. Therefore, veterinary drugs might also be a problem for the environment not only due to their presence in the wastewater but also to sludge biosolids reuse and through particulate matter sedimentation.

Sludge reactors with $100 \mu\text{g L}^{-1}$ initial drug charge presented removal rates of 68% for ENR and 76% for TET after a 10-days period. These values represent still considerable concentrations at $\mu\text{g L}^{-1}$ level that remain in the aqueous phase and consequently will pass through the WWTP to the receiving environment. The risks associated with effluents containing trace pollutants such as antibiotics remains uncertain. In addition, conventional activated sludge treatment is characterized by shorter hydraulic and solid retention times, indicating that there will be less opportunity for veterinary pharmaceuticals to degrade.

This study also emphasizes the fact that measuring only the dissolved fraction of pharmaceuticals in the WWTP effluent may underestimate the loading and risks to the aquatic environment, particularly for highly organic loaded wastewater such as those of livestock industry.

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Chapter 7 - Potential of *Phragmites australis* for the removal of veterinary pharmaceuticals from aquatic media

7.1 Introduction

7.2 Experimental layout

7.2.1 Nutrient solution experiments

7.2.2 Wastewater experiments

7.3 Results and discussion

7.3.1 Nutrient solution experiments

7.3.2 Wastewater experiments

7.4 Conclusions

References

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7.1 Introduction

CWs and natural wetland systems are gaining attention as water quality improving systems as alternative or additive low-cost wastewater treatments (Dordio et al. 2010, Meers et al. 2005). The widespread use of some drugs and their generally inefficient removal from wastewaters in WWTPs, the main reasons for the frequent detection of pharmaceuticals in aquatic bodies, led to a growing need for alternative wastewater treatment processes for removing pharmaceuticals from waters

Although CWs are widely used for municipal wastewater, its application to agricultural or industrial wastewater still poses challenges once wastewater composition variability and treatment needs can be very specific (Calheiros et al. 2007). The majority of works focused on this topic (Dordio et al. 2009, Hijosa-Valsero et al. 2010, Llorens et al. 2009, Zhang et al. 2011) are specific to human pharmaceuticals, as discussed in Chapter 1. Regarding veterinary drugs, there are only two articles, one focused on the removal of sulfonamides (veterinary antibiotics) from swine wastewater by a constructed macrophyte floating bed system (Xian et al. 2010) and another focused on ionophores (pharmaceuticals used exclusively for veterinary application considered as high-risk compounds) removal by a mesocosm scale FWS CWs (Hussain et al. 2012).

The aim of the present work was to evaluate the capacity of *P. australis* to remove veterinary pharmaceutical compounds, ENR, CEF and TET from aquatic mediums, including wastewater from livestock and slaughterhouse industries. The widely use of *P. australis* in CWs design in Europe, including Portugal (Calheiros et al. 2007), led to the selection of this plant.

7.2 Experimental layout

Material and reagents, samples collection of both plant material and wastewater, analytical methodologies and experiment set-up are detailed on Chapter 2.

Plants fresh biomass was accessed at the beginning and at the end of the experiments, ranging the individual plant total fresh weigh from 7 to 13 g. At the end of the experiments plants were air dried until constant weight and separated into shoots and roots. Roots accounted for ca. 7% of plant total weight.

Method recovery percentages were 86, 87 and 80 % for TET, ENR and CEF, respectively, and the overall variability of the method was below 14 %. The LODs in this work, due to a higher volume of sample used (250 mL) for both nutrient solution and wastewater, were 0.4, 0.2 and 0.6 $\mu\text{g L}^{-1}$ for TET, ENR and CEF, respectively.

7.2.1 Nutrient solution experiments

Nutrient solution was initially chosen as preliminary testing conditions, once it allows to have an aquatic medium with the essential nutrients for plant growth and simultaneously be less prone to matrix interferences and less prone to condition drug bioavailability, such as expected for wastewater. Depending on its characteristics the wastewater may also lead to plants stress or even dieback due to toxicity (Han et al. 2011).

Plants used, 3 by flask, accounted for 27 ± 6 g of fresh biomass, being 2.0 ± 0.4 g due to roots.

The time of the experiment chosen was based on the literature and had into account the hydraulic retention times normally used for micro and mesocosms studies as well as in full scale CWs (Weber and Legge 2011, Zhang et al. 2008). Drug concentrations selected represent real concentrations previously found for veterinary drugs, e.g. ENR in effluents from WWTPs from pharmaceutical industry (Babić et al. 2010) and oxytetracycline in swine wastewaters (Ben et al. 2008).

Once the behavior of drugs under the experimental conditions was unknown, especially the kinetics and the percentage of removal, either with or without plants, doping in 2 days cycles was selected.

7.2.2 Wastewater experiments

Livestock (swine) wastewater used presented pH of 7.63, COD of 337.5 mg L^{-1} and 48.15 g L^{-1} of particulate matter being 99% organic particulate matter.

Plants used, 4 by flask, accounted for 44 ± 6 g of fresh biomass, being in average 3.2 ± 0.9 g due to roots.

7.3 Results and discussion

7.3.1 Nutrient solution experiments

Enrofloxacin concentration (Table 10) increased in the solutions of both control and SWP samples from day 1 to day 7, as expected due to the periodic doping of the solution (in days 0, 2, 4 and 6). For control samples (solution without plants) ENR concentrations were identical or only slightly lower than the doping values (concentration decreased between 0 and 25%) indicating a relatively low natural degradation of the drug, which was more evident

after 7 days. The concentration decrease could be related for instance with drug adsorption to the vessels and/or photodegradation. For solution with plants (SWP) samples, for initial concentration level of $10 \mu\text{g L}^{-1}$, at day 1 and 4 ENR could not be detected whereas at day 7 ENR concentration was 85% lower than the expected level ($40 \mu\text{g L}^{-1}$). For the initial level of $100 \mu\text{g L}^{-1}$, ENR concentration in solution decreased between 67 and 91% from the expected levels. The significant ($p < 0.05$) difference in ENR concentrations observed in the presence and in the absence of plants denotes a positive effect of *P. australis* on ENR degradation.

Table 10 Enrofloxacin (ENR), ceftiofur (CEF) and tetracycline (TET) concentrations (mean values \pm standard deviation ($n=3$)) observed over time for the experiment carried out in nutrient solution (initial levels¹ of 10 and $100 \mu\text{g L}^{-1}$). Results for solution with plants (SWP) and control samples without plants (Control) are presented².

	Initial level $10 \mu\text{g L}^{-1}$			Initial level $100 \mu\text{g L}^{-1}$		
	day 1	day 4	day 7	day 1	day 4	day 7
ENR						
SWP	<LOD ³	<LOD ³	6 ± 2^a	11.8 ± 0.8^a	19 ± 4^a	133 ± 2^d
Control	10.3 ± 0.8^a	14 ± 9^a	32.0 ± 0.5^b	79 ± 3^b	202 ± 15^c	302 ± 18^e
CEF						
SWP	9.9 ± 0.6^a	11.8 ± 0.4^a	24 ± 5^b	73 ± 13^a	148 ± 11^b	140 ± 26^b
Control	11 ± 2^a	$17 \pm 3^{a,b}$	38.1 ± 0.6^c	87 ± 18^a	191 ± 11^c	$178 \pm 34^{b,c}$
TET						
SWP	1.11 ± 0.02^a	$6.8 \pm 0.3^{b,c}$	11 ± 4^c	17 ± 3^a	24 ± 7^a	70 ± 30^b
Control	$4.0 \pm 0.1^{a,b}$	$4.0 \pm 0.6^{a,b}$	$3.5 \pm 0.7^{a,b}$	27 ± 2^a	34 ± 1^a	48 ± 4^a

¹ Flasks were doped periodically at day 0, 2, 4 and 6 making the concentrations range from 10 to 40 and from 100 to $400 \mu\text{g L}^{-1}$.

² Same letters indicate results statistically identical ($p > 0.05$) within each drug level.

³ lower than method detection limit (LOD = $0.2 \mu\text{g L}^{-1}$)

For both ENR concentrations under study (initial level of 10 and of $100 \mu\text{g L}^{-1}$), a similar behavior of the solutions pH was observed. Control samples presented a stable pH value of ca. 4, whereas SWP samples showed an increase in pH, from 4 to 8, during the trial. This pH trend was similar to the one reported for a study on human pharmaceuticals uptake by plants in fortified nutrient solution, where pH increased 3 units over the course of the study (Herklitz et al. 2010).

To investigate if the pH of the solution could be important in the natural degradation of ENR, further experiments (nutrient solution with ENR but without plants – control experiment) were carried out in parallel at both pH of 4 and 8 (the pH that SWP samples had after 7 days of experiment). Obtained results (e.g. 269 ± 11 and $283 \pm 9 \mu\text{g L}^{-1}$ for pH 4 and 8 respectively

at the higher doping level) were statistically identical indicating that there was no influence of the pH. Therefore, the ENR removal percentage observed in SWP samples was mainly due to the presence of the plant.

Exudate solutions (not doped nutrient solution in which the plants were placed after being exposed to the doped solutions) presented concentrations of ENR ranging between <LOD and $0.8 \mu\text{g L}^{-1}$ (plants previously exposed to the initial level of $10 \mu\text{g L}^{-1}$) and between 4 to $18 \mu\text{g L}^{-1}$ (plants previously exposed to the initial level of $100 \mu\text{g L}^{-1}$). These ENR residual levels in exudate solutions (that represent only at most 10% of the amount of drug removed/degraded in the flasks with plants during the 7 days experiments) may result of ENR adsorption to plant roots during the previous drug exposure period or of plant exudation of absorbed ENR.

Ceftiofur concentration (Table 10) also presented an increase in solution from day 1 to day 7, which was more pronounced for control than for SWP samples. However, for the higher initial level ($100 \mu\text{g L}^{-1}$) CEF concentration stabilized from day 4 to 7. As observed for ENR, this drug suffered also some natural degradation after 7 days, particularly noticeable for the higher initial level, with a 56% decrease in the CEF concentration in control samples solutions. Overall, SWP samples presented lower CEF concentrations than the correspondent control samples (although differences were not always statistically significant). The positive effect of *P. australis* in CEF removal was not as evident as for ENR.

For CEF, control and SWP samples presented very small pH variations (lower than 0.5 pH units, initial pH 4) during the 7 days period, contrary to what was observed for ENR.

Exudate solutions presented concentrations of CEF ranging between <LOD and $1.4 \mu\text{g L}^{-1}$ and between <LOD and $6 \mu\text{g L}^{-1}$ (plants previously exposed to the initial level of $10 \mu\text{g L}^{-1}$ and of $100 \mu\text{g L}^{-1}$, respectively), indicating that during the previous period of drug exposure, CEF was either adsorbed or absorbed by the plant roots. These residual levels represent, as for ENR, only at most 10% of the amount of drug removed/degraded in the flasks with plants being in both cases not correlated with the period of the study (day 1, 4 or 7) or with the concentration levels.

Overall results for TET (Table 10) showed high concentration decreases, for both drug levels studied. These significant decreases (between 60 and 98% from the expected levels) were in some cases higher in control than in SWP samples.

The drug TET presented also a distinct pH behavior from the previous ones. Solutions of all control samples presented a stable pH value around 4, whereas SWP samples showed an increase in pH (ca one unit) during the 7 days experiment.

Taken as a whole, a positive effect of *P. australis* on TET drug removal was not evident. Although in some cases SWP samples presented lower TET concentrations than the correspondent control samples no clear pattern could be observed. Antibiotic compounds of the tetracycline class are known to be particularly unstable upon exposure to light (Werner et al. 2006) and for this set of experiments (nutrient solution) flasks were not protected from light. In fact, further tests with flasks (without plants) exposed and not exposed to light

revealed a significant effect of natural light on TET natural degradation (e.g. 98% of removal from solutions exposed to light vs. 76% of removal from solutions not exposed to light). In addition, tetracycline antibiotics have a high adsorption tendency to solid matrices (Halling-Sørensen et al. 2002). Therefore, in the presence of plants TET may have been adsorbed to plant roots making it less prone to photodegradation.

Exudate solutions presented concentrations of TET ranging between 3 and 5 $\mu\text{g L}^{-1}$ and between 4 and 13 $\mu\text{g L}^{-1}$ (plants previously exposed to the initial level of 10 $\mu\text{g L}^{-1}$ and of 100 $\mu\text{g L}^{-1}$, respectively). This result confirms that a percentage of TET removal from the medium is due to an active role of the plant.

Nutrient solution experiments demonstrated that the plant *P. australis* can play an important role in the drugs removal and/or degradation from the aquatic medium. In spite of the differences observed among ENR, CEF and TET, at both concentration levels tested, the medium with plants presented in general lower levels than the medium without plants. Differences in the SWP samples behavior may be related not only with the drug itself but also with the phenological cycle of the plant and with the consequent plant activity once drug tests were performed separately in different dates. It is already known that temperature can be important for the elimination of some pharmaceuticals in constructed wetlands (Hijosa-Valsero et al. 2011), in agreement with previous observed relation between removal efficiencies and seasonality (summer/winter comparison) (Dordio et al. 2010). Different plant activity may affect plants exudation and further the aqueous medium as observed by the different pH variations.

It was expected that microbial degradation had a very limited contribution, given the care taken to diminish the microbial populations in the medium when the assays were setup. In fact natural degradation of ENR and CEF was reduced and the higher natural degradation of TET was probably associated with photodegradation. Therefore, drug removal by the plants resulted, mainly, from adsorption on the roots, plant uptake and degradation. Pharmaceuticals octanol-water partition coefficient ($\log K_{ow}$) is a property commonly used to evaluate if the compounds have adequate properties to move through cell membranes and enter the plant's transpiration stream. Organic compounds with $0.5 < \log K_{ow} < 3$ are considered easily taken up by the plants (Dordio et al. 2010). For the current veterinary drugs ENR, CEF and TET $\log K_{ow}$ are 2.3, 0.3 and -1.3 respectively, being ENR and CEF more probable to be suitable for plant uptake. In addition, it has been verified that some human pharmaceuticals commonly present in treated wastewater and biosolids can be actively taken up by various species of plants grown in nutrient solutions fortified with pharmaceuticals under ideal hydroponics conditions (Herklotz et al. 2010). As for veterinary pharmaceuticals, literature already presents some plants interaction with these drugs but from the point of the existence of possible adverse effects on manure application in soil (Boxall et al. 2006, Dolliver et al. 2007, Kumar et al. 2005).

7.3.2 Wastewater experiments

The role of *P. australis* on veterinary drugs degradation and/or removal was further studied using a real pig farm treated wastewater. Once the plant presented no visual damage after exposure to drugs in the nutrient solution experiments, the most unfavorable conditions, the higher concentration level of $100\ \mu\text{g L}^{-1}$ and a 7 days period of exposure, were selected. Since, in general, natural degradation observed in the nutrient solution trials was low, only the initial doping was performed.

Although plants have the ability to degrade or sequester many toxic compounds they can also be sensitive to many of them. In fact, the stress response of plants to the presence of pollutants can influence plants capacity to control the uptake of those pollutants, increasing the uptake and sometimes causing serious problems to the viability of the plant (Almeida et al. 2008 and references therein). In addition, pharmaceuticals are known to have phytotoxic potential, namely for crop plants grown on contaminated soil/manure (Farkas et al. 2009, Herklotz et al. 2010). Information available in the literature is not clear, however, if the negative effects on plants are caused by the pharmaceuticals themselves or if the antimicrobial action of pharmaceuticals affects soil microorganisms, affecting the plant-microorganism symbiosis (Fatta-Kassinos et al. 2011). In addition, the wastewater by itself can be toxic to the plants (Han et al. 2011). This combined phytotoxic potential can represent a problem for phyto-WWTPs.

In this study chlorophyll contents (chlorophyll a, b, and total chlorophyll content) of the leaves of the plants used in the experiment were determined as a measure of possible induced stress caused by wastewater and/or pollutants. This parameter was also used to evaluate possible damage induced by other organic xenobiotics (Dordio et al. 2009) and references therein). Chlorophyll contents in plants leaves (e.g. 1.30 ± 0.08 and 1.65 ± 0.08 mmol of total chlorophyll g^{-1} (fresh weight) in nutrient solution and wastewater control, respectively) (Figure 25), denote the absence of stress induced by the wastewater. On the other hand, this parameter pointed to some plant stress due to exposure to the veterinary drugs (e.g. 1.65 ± 0.08 , 1.2 ± 0.4 , 1.1 ± 0.1 and 1.1 ± 0.2 mmol of total chlorophyll g^{-1} (fresh weight) in wastewater control, ENR CEF and TET samples, respectively).

Regarding pH, solutions presented slight pH variations (ca. 0.5 pH unit) among the different solutions in comparison with the initial conditions.

Drugs under study presented distinct behaviors among themselves (Figure 26). After 7 days, CEF could not be detected in any of the samples, probably due to strong binding between the drug and the high organic content of the wastewater matrix. In fact, in the previous study carried out with activated sludge a complete remove of CEF from solution was also observed (Chapter 6). The partition of CEF may have led to retention of the compound in the organic solid phase during samples filtration and, as consequence, to undetectable levels of CEF on the soluble aqueous phase. Therefore, for this drug the positive effect that the plant might have on its removal was not confirmed.

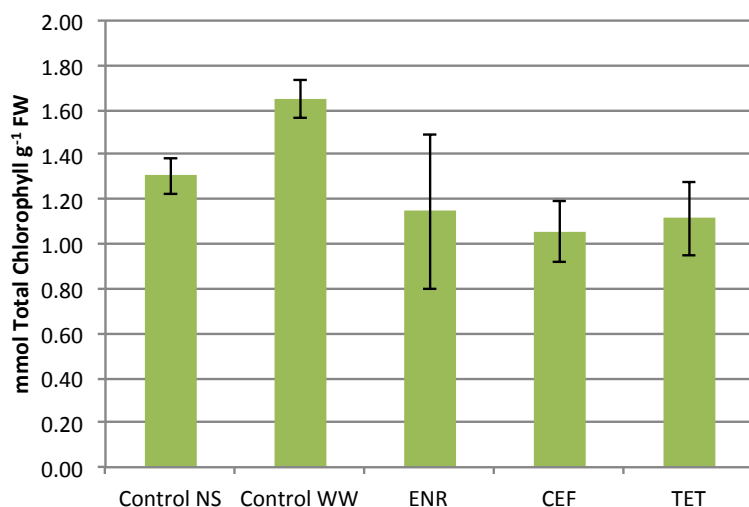


Figure 25 Total chlorophyll content expressed as mmol per gram of leaves fresh weight (FW) of *Phragmites australis* collected from the flasks of nutrient solution (control NS), wastewater (control WW), enrofloxacin (ENR) doped wastewater, ceftiofur (CEF) doped wastewater and tetracycline (TET) doped wastewater.

The ENR samples presented levels of $6 \pm 2 \mu\text{g L}^{-1}$ for SWP and $43 \pm 5 \mu\text{g L}^{-1}$ for control samples after 7 days, resulting in 94% and 57% of drug removal, respectively. These results corroborate those observed in the experiments carried out in nutrient solution. The higher removal efficiencies, comparatively to that observed in nutrient solution experiment may also be due to some partition of ENR into the suspended organic matter of the wastewater. This partition can justify a lower ENR concentration in the soluble fraction, which leads to the

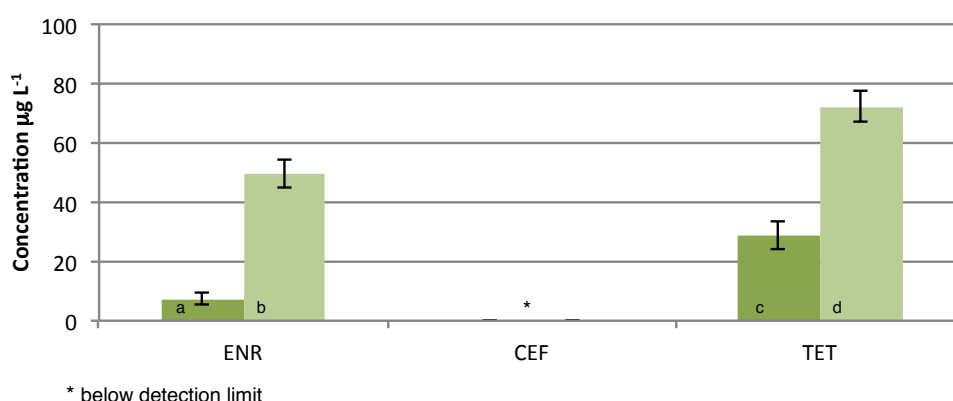


Figure 26 Drug, enrofloxacin (ENR), ceftiofur (CEF) and tetracycline (TET) concentration levels observed in wastewater after a 7 days period. Initial level of each drug: $100 \mu\text{g L}^{-1}$ (■ wastewater with plants; □ wastewater without plants). Different letters indicate results statistically different ($p < 0.05$).

suggestion of a slightly higher natural attenuation.

Tetracycline levels in wastewater after the 7-day trial period ($25 \pm 5 \mu\text{g L}^{-1}$ for SWP and $62 \pm 5 \mu\text{g L}^{-1}$ for control samples after 7 days) indicated a 75% and 38% of drug removal, for SWP and control samples, respectively. In this case, the natural attenuation was much less extent than that observed for the nutrient solution experiment, because flasks were light protected to decrease the known TET photodegradation. Therefore, results observed for wastewater denote the positive effect of the plant on TET degradation/removal from the medium.

Levels found in control samples for both ENR and TET drugs were statistically different from those in SWP samples, being observed a significantly higher drug removal and/or degradation in solution with plants. Correlation between the plants biomass in each flask and the respective percentage of drug removal from the wastewater was investigated. Results (Figure 27) revealed a significant correlation ($r^2 = 0.7919$, $p < 0.05$, $n = 6$), indicating that higher biomass usage should improve drug removal from the medium.

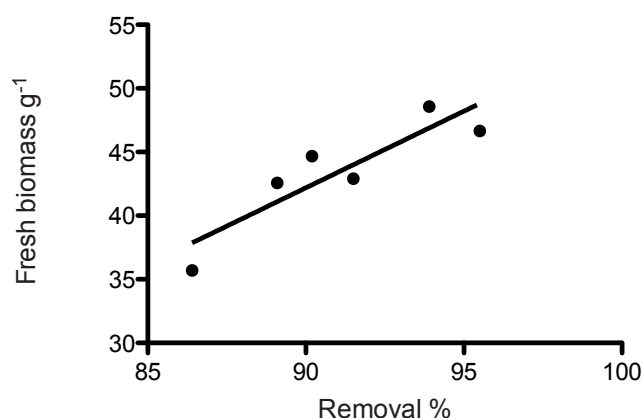


Figure 27 Correlation between plant fresh biomass in each flask and the respective percentage of drug removal (enrofloxacin (ENR) and tetracycline (TET)) from the wastewater. Results revealed a significant correlation ($r^2 = 0.7919$, $p < 0.05$, $n = 6$).

Macrophytes can contribute directly or indirectly to pollutant removal in CWs. Besides the direct processes (uptake, accumulation, degradation, phytovolatilization, adsorption and the release of plant exudates), indirect processes related to biofilm growth around roots and in the case of CWs to the pumping of oxygen towards the rhizosphere, thus changing redox conditions (Hijosa-Valsero et al. 2011). The biota that are found in wetlands support the essential structures and processes. Combined effects of the physical, chemical and biological characteristics of a wetland, including microbial, plant and animal communities, is required to understand its hydrological and biogeochemical functioning (Harrington and McInnes 2009).

So another factor has to be taken in consideration in drugs removal/degradation, which is the wastewater microbial population. For this experiment, while plants were washed with Micropur to remove native microbial community attached to their roots, wastewater microbial community was unchanged. The association of both microorganisms and plants can also be of tremendous importance in the process of drugs elimination from aqueous samples.

To investigate the potential effect of the microorganisms present in the medium, microbial abundance was estimated as the total cell counts (TCC) of total DAPI-stained cells (Figure 28). TCC in the initial wastewater (8.0 \log_{10} TCC mL^{-1}) was significantly higher than in the remaining samples at the end of the experiment, pointing to a decrease of the number of microorganisms with time. After 7 days, TCC of solutions with drugs, either with (P) or without plants (C), were in general significantly lower than those without drugs (with (Control 1) and without plants (Control 2)). This difference between solutions with and without drugs indicates a probable toxic effect of the drugs in the medium that led to the decrease of the microbial abundance. On the other hand there were in general no differences in TCC between solutions with and without plants, which indicates that microorganisms probably did not have a significant contribution on drugs removal and/or degradation. However, these results correspond only to a 7-day period of experiment without any support matrix, while in a real system operating continuously more influence of the microbial community on drug removal/degradation could be expected.

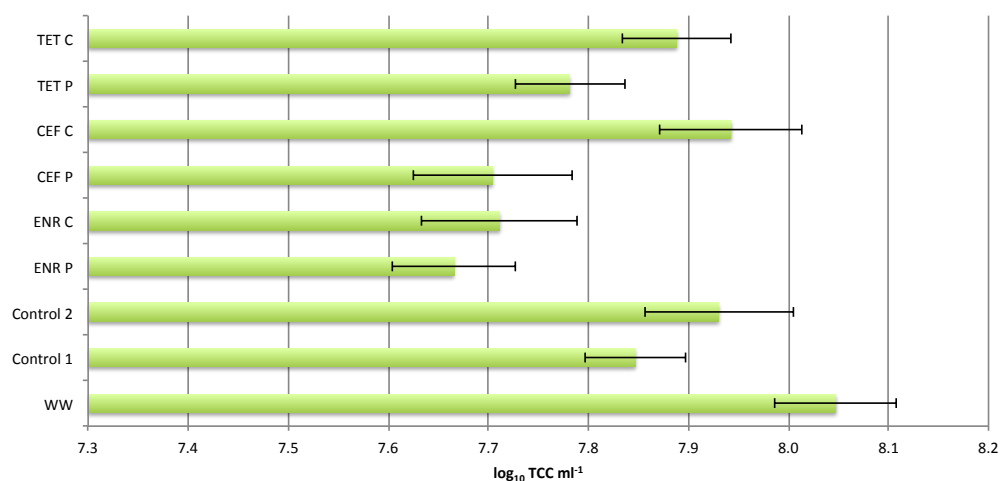


Figure 28 Total cell counts (TCC) of total DAPI-stained cells in the initial wastewater (WW) and in the samples of wastewater after a 7-days period, without (C) and with (P) plants and with or without (Control 1 and Control 2) drugs.

Adsorption samples (deionized water in which the plant roots were washed after exposure to the wastewater doped with each drug) revealed quantifiable levels of ENR, $0.8 \mu\text{g L}^{-1}$, and TET, $3 \mu\text{g L}^{-1}$, whereas CEF once again could not be detected. These residual levels of drugs in adsorption samples were similar to those obtained in the nutrient solution experiments for exudate solutions. On the other hand, in this experiment, all the exudate solutions did not reveal detectable levels of any of the drugs under study. Therefore, these results indicate that a small quantity (3.4% or lower) of the drug removed by the plants was probably adsorbed to the root. These last results also indicated that the percentage of removal of the drugs by adsorption to the roots was low, and that the main mechanisms occurring should be drug removal by plant uptake and/or degradation in the roots area.

Therefore, present work indicated that *P. australis* has potential to remove veterinary drugs from wastewater. Taking in consideration that this plant is one of the most used in phyto-WWTPs, these “green” systems can be an additive low-cost wastewater treatment for wastewater of livestock and slaughterhouse industries contributing for the elimination of veterinary pharmaceuticals.

Application of a constructed macrophyte floating bed system with three varieties of Italian ryegrass (*Lolium multiflorum* Lam.) to improve the water quality of swine wastewater has already provided good results, not only to the reduction of nutrients levels and chemical oxygen demand (COD), but also for reduction of sulfonamide antimicrobials (Xian et al. 2010). On the other hand, the support matrix is also a very important component in CWs not only to support the growth of macrophytes and microorganisms but can also promote a series of chemical and physical processes that may improve wastewater treatment. Sorption by the solid matrix is proven to play an important role in contaminant retention (Dordio et al. 2010). So, more studies on this subject are in demand. However, veterinary drugs toxicity for the CWs plants must not be forgotten. For instance, phytotoxicity of enrofloxacin on crop plants (*Cucumis sativus*, *Lactuca sativa*, *Phaseolus vulgaris* and *Raphanus sativus*) is known to generate both toxic effect and hormesis, related to plant drug uptake (Fatta-Kassinos et al. 2011). Further studies should be performed at a pilot scale to evaluate the effective potential of CWs for drug removal and effects on plants of macrophyte bed. Also, the possible generation of intermediates more resistant to degradation and with the characteristics of exhibiting equal or more toxic effects than the parent compounds must be considered as well. Especially, when considering wastewater reuse, intense research must be launched towards this direction to safeguard environmental ecosystems.

7.4 Conclusions

The plant *P. australis* played an important role in drugs removal from the aquatic medium. Microbial abundance estimated revealed in general no differences between solutions with and without plants, indicating that microorganisms were not a major participant in drugs removal and/or degradation. Occurrence of adsorption of the drugs to plant roots was observed, however such adsorption was low. Therefore, mechanisms occurring for veterinary drugs removal were plant uptake and/or degradation.

The present results show the potential positive effects of *P. australis*-planted beds to be used in CWs for the reduction/elimination of veterinary pharmaceuticals from livestock and slaughterhouse industries wastewater.

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Chapter 8 - Potential of constructed wetlands microcosms for the removal of veterinary pharmaceuticals from wastewater

8.1 Introduction

8.2 Experimental layout

8.2.1 Microcosms operation and sampling

8.3 Results and discussion

8.3.1 Planted versus unplanted systems

8.3.2 Systems performance throughout time

8.4 Conclusions

References

This chapter is in submission process

8.1 Introduction

The widespread use of some pharmaceuticals and their generally inefficient removal in WWTPs led to a growing need for alternative wastewater treatment processes for removing them from wastewaters as discussed in Chapter 1. CWs and natural wetland systems can be managed as water quality improving systems, as alternative or additive low-cost wastewater treatments (Dordio et al. 2010, Meers et al. 2005).

The research on the ability of CWs to reduce pharmaceuticals concentrations in wastewaters has been focused mainly on human pharmaceuticals (Dordio et al. 2009, Hijosa-Valsero et al. 2010, Llorens et al. 2009, Matamoros and Salvadó 2012, Park et al. 2009, Zhang et al. 2011) being, in addition, the information limited to only a few compounds and plant species. Regarding veterinary drugs, to our knowledge there is a scarcity of studies with only 2 articles recently published on this subject (Hussain et al. 2012, Xian et al. 2010). Therefore, more research on this topic is in need.

The aim of the work described in this chapter was to evaluate, at microcosm level, the capacity of CWs to remove two veterinary pharmaceutical compounds, ENR and TET, from livestock industries wastewater. CEF was not included in this study once the previous results both on batch reactors (Chapter 6) and plant microcosms (Chapter 7) were inconclusive. Microcosms were planted with *P. australis*, the plant previously studied (Chapter 7) that presented positive effects in the reduction/elimination of these veterinary drugs from livestock wastewater.

While full-scale wetland studies are valuable for assessing overall removal efficiencies, identifying and quantifying the important removal processes for emerging organic pollutants is complicated by the complexity of interactive processes present in wetlands. Consequently, microcosm scale studies are crucial to differentiate processes, particularly with regards to those associated with plants (Reinhold et al. 2010). The microcosms approach has been shown to suitably represent interactions among plants, microorganisms, substrates and contaminants within a complex rhizosphere system (Helt et al. 2012) and was the approach selected in this work.

8.2 Experimental layout

Material and reagents, samples collection of both plant material and wastewater, analytical methodologies and experiment set-up are detailed on Chapter 2.

Analytical methodology used for wastewater analysis was developed for a relatively low volume of wastewater (Chapter 4). In this work, due to a higher volume of sample used (250 mL), the LODs were 0.4 and 0.2 $\mu\text{g L}^{-1}$ for TET and ENR, respectively. Sediment samples

were analyzed accordingly the developed methodology (Chapter 5), being the results obtained corrected by the recovery percentage of the drug.

8.2.1 Microcosms operation and sampling

Initial wastewater presented pH of 8.04, COD of 1042 mg L⁻¹ and 340 mg L⁻¹ of particulate matter being 82% organic particulate matter. Variations in wastewater characteristics were considered negligible along time due to the dimension of the lagoons (buffer effect). pH (the only parameter systematically evaluated) variation was low (< 0.6 units) throughout the study.

Plants fresh biomass was accessed at the beginning and at the end of the experiments. The density of ca. 40 plants per microcosm used (Chapter 2) granted plants fresh biomass ranging from 902 to 914 g. At the end of the experiments (after 12 weeks) plants were air dried until constant weight and separated into shoots and roots. Plant total biomass increased between 10 and 20 %. Roots accounted for ca. 8% of plant total dry weight, rhizomes for ca. 17%, leaves for ca. 19% and stems for ca. 56%.

The conditions chosen in this experiment in terms of time period (HRT) and drugs concentrations were those selected in the studies of the previous Chapter 7 by the reasons mentioned there.

8.3 Results and discussion

8.3.1 Planted versus unplanted systems

All systems tested presented a significant reduction in drug concentration in solution, along all 1-week cycles, for both ENR and TET. In general, no significant differences were observed among drug levels in the final solutions. Final levels obtained (Figure 29) indicate at least 94% removal of TET (final level < 6 µg L⁻¹) and 98% removal of ENR (final level < 2 µg L⁻¹) from solution relatively to the 100 µg L⁻¹ doping level, after 1 week, in the microcosms. Comparing, for each drug, planted (EP or TP) vs. unplanted systems (EX or TX), there were no significant differences between the microcosms denoting the crucial influence of the substrate on drug removal from the wastewater. Previous results (Chapter 7) have demonstrated that the plant *P. australis* can play an important role on ENR and TET removal from wastewater. However, in this study, plant direct influence could not be verified once planted and unplanted systems did not present significant differences. Therefore, main mechanism occurring for drug removal was probably adsorption and/or microbial degradation in the microcosms' substrate.

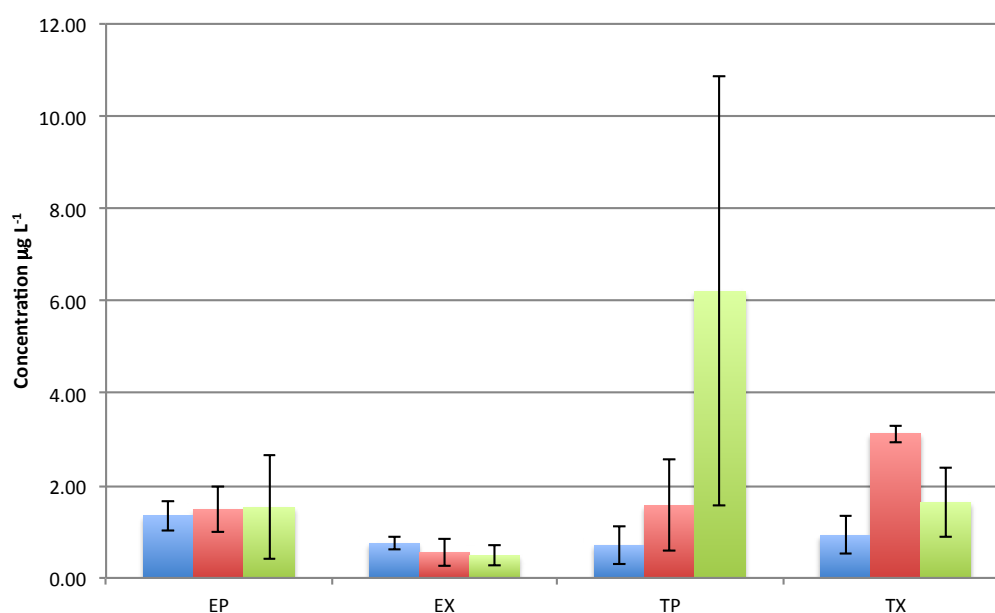


Figure 29 Drug concentration in solution (mean values, n=6) obtained for the enrofloxacin planted (EP) and unplanted (EX), as well as tetracycline planted (TP) and unplanted (TX) microcosms after 1; 2; 4 weeks.

Sterilized microcosms analysis resulted in quantifiable levels of ENR ($0.2 - 4 \mu\text{g L}^{-1}$) and TET ($5 - 22 \mu\text{g L}^{-1}$) in solution within the same period. These results showed similar levels for ENR in solution from unplanted and sterilized microcosms revealing that ENR removal in microcosms' beds was due to adsorption of the drug to the system. For TET, levels in solution from the sterilized microcosms were significantly higher than those from unplanted microcosms, indicating that the microbial community present on both planted and unplanted microcosms may be degrading the compound. In the root bed substrate (the top layer of the microcosm with an expected affinity for drug adsorption due to sediment high fine fraction and high organic matter content) none of the drugs was detected either in planted or unplanted systems along time.

The additional test performed using the unplanted microcosm systems just filled with gravel fractions (gravel plus lava rock without sediment) showed that for both ENR ($0.8 - 2 \mu\text{g L}^{-1}$) and TET ($1 - 2.5 \mu\text{g L}^{-1}$) removal efficiencies from solution were similar to those for microcosms systems with the roots' bed substrate and lower than those for the sterilized unplanted systems. Therefore, these results show that, also for the gravel fraction, adsorption and/or degradation of the drugs occur within the 1-week cycles. This removal could also be responsible for the non-detected drug levels in the root bed substrate. The microcosms assembled had three different layers that could all contribute for the removal of the drug from the solution. The selection of a medium with a high sorption capacity can be an important step in the optimization of the CWs performances (Dordio et al. 2007). However, the sorption of any

compound to a fixed amount of solid matrix is not infinite. Thus, it is vital to understand the sorption of such compounds to the substrate within a treatment wetland over the long term (Conkle et al. 2010).

Toxicity tests were performed to evaluate CWs improvement of treated wastewater toxicity. Results from ToxScreen tests showed that all samples both treated wastewater and sediment elutriates induced reduction in bacterial luminescence. Initial wastewater added to the microcosm systems without and with drug addition (both ENR and TET) presented toxicity higher than 99.9%. Results (Figure 30) show a decrease in wastewater toxicity after treatment. Unplanted microcosms tend to result in lower toxicity after each 1-week cycle of treatment than the respective planted one. Sediment elutriate results show a trend for lower toxicity in sediments after treatment in the planted systems than in the unplanted ones. These last results may result from a possible higher degradation of the compounds present in the initial wastewater in the planted substrate and/or from a stronger adsorption to sediments in the unplanted ones. However, results clearly indicate that the CWs microcosms reduce significantly the toxicity of the wastewater, toxicity that was independent of the presence or not of the drugs.

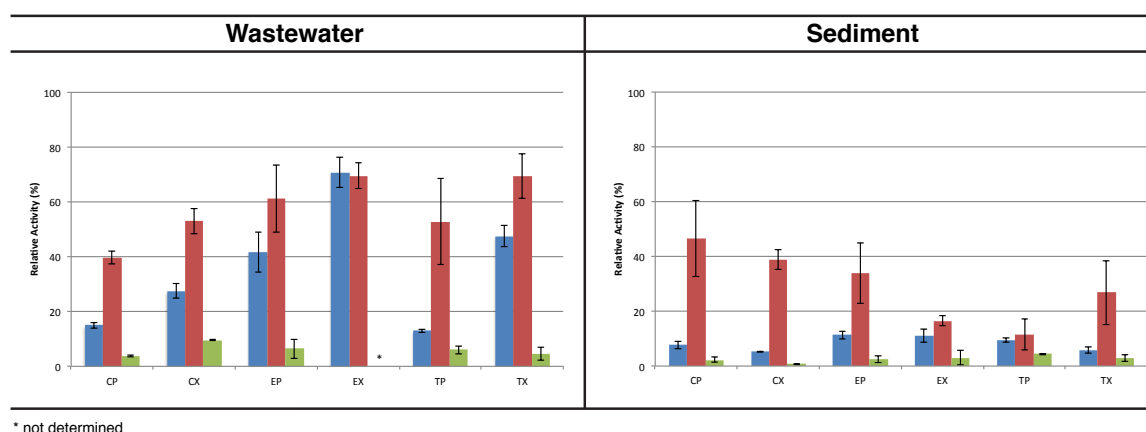


Figure 30 ToxScreen tests measured as relative activity (%) (mean values, n=3) in wastewater and sediment elutriates along the first 4 weeks study for the different samples, control planted (CP), control unplanted (CX), enrofloxacin planted (EP), enrofloxacin unplanted (EX), tetracycline planted (TP) and tetracycline unplanted (TX), after 1; 2; 4 weeks.

The pH results obtained (Figure 31) confirm that after four weeks, the systems were differentiated according to their treatment, being the values statistically different among type of treatment. Unplanted microcosms (CX, EX and TX) pH increased until it reached the higher value, the pH of the initial wastewater 8.05 ± 0.05 , whereas in planted ones (CP, EP, TP) pH values remained around 7.5. Sterilized microcosms (ES and TS) had the lower pH.

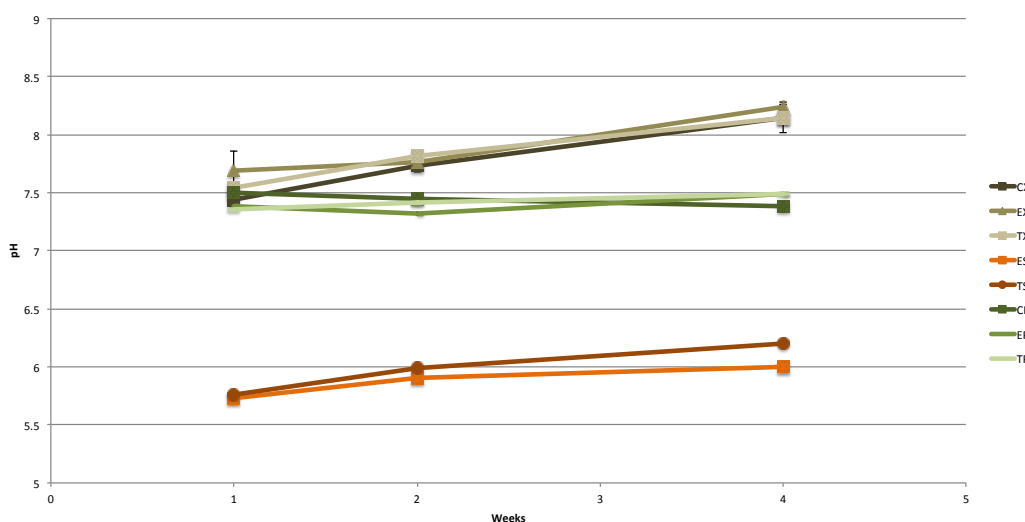


Figure 31 pH values determined along the first 4 weeks for the different solution samples: control unplanted (CX), enrofloxacin unplanted (EX), tetracycline unplanted (TX), enrofloxacin sterilized (ES), tetracycline sterilized (TS), control planted (CP), enrofloxacin planted (EP), tetracycline planted (TP).

Depuration in CWs is achieved by the concerted action between plant roots and rhizomes, microorganisms and the solid media components. These treatment systems provide different microenvironments where various removal processes can take place: physical (retention, adsorption on the substrate, adsorption on the biofilm, photodegradation, volatilization), chemical (degradation), vegetal (plant uptake, phytovolatilization, release of exudates, oxygen pumping to the rhizosphere, providing an adequate surface for biofilm growth) or microbiological (metabolization). Drugs photodegradation can be discarded due to microcosms setup as well as volatilization due to compounds characteristics (high molar masses and non-volatile). Vegetal processes may occur not from direct action of the plant in the drugs, but by influence of the rhizosphere.

Current results show potential of CWs to be used for the reduction/elimination of veterinary pharmaceuticals from livestock and slaughterhouse industries wastewater. Occurrence of adsorption of the drugs to the substrate may be the predominant mechanism for ENR, although for TET there were signs that degradation is also occurring. The plant appears not to have a direct effect on drugs removal. However, planted microcosms presented lower clogging problems along the study, they lasted eight more weeks. In addition, plants mediate important processes in CWs, for example, plant metabolic activity releases oxygen into the rhizosphere, which aids in nitrification through the direct uptake of nutrients. For livestock effluents, absorption of inorganic nitrogen and phosphorus and conversion into plant biomass (Adhikari et al. 2011) is also of extreme interest when considering CWs application as a multi-task polishing step.

8.3.2 Systems performance throughout time

Once microcosms were only left to acclimate for one week, plants and microbial community could be in adaptation period along the previously analyzed four weeks. Microcosms set-up was intended to continually run for the possible longer time period. It is known that temperature and its relation with seasonality (summer/winter comparison) can be important for the elimination of some pharmaceuticals in CWs (Dordio et al. 2010, Hijosa-Valsero et al. 2011). In addition, adsorption to the substrate as main mechanism may lead in the long term to substrate saturation being important to evaluate systems performance throughout time. However, unplanted systems clogged at the sixth week and only the planted ones could be tested throughout time until their clogging at the thirteenth week. So, results presented for planted microcosms were obtained after a twelve weeks period.

Planted systems presented similar results along the twelve weeks of study. Final levels in solution after each one-week cycle (Figure 32) indicate at least 94% removal of TET (final level $< 6 \mu\text{g L}^{-1}$) and 98% removal of ENR (final level $< 2 \mu\text{g L}^{-1}$) of the $100 \mu\text{g L}^{-1}$ doping level. Removal efficiencies were very stable for ENR and TET microcosms, except of one point for TET possible due to punctual analytical errors. These results show that CWs provide an efficient and reproducible methodology along time for these veterinary drugs removal from wastewater. Due to clogging, systems could only be evaluated along a summer season, lacking in data for a winter season to check for possible influences of the temperature.

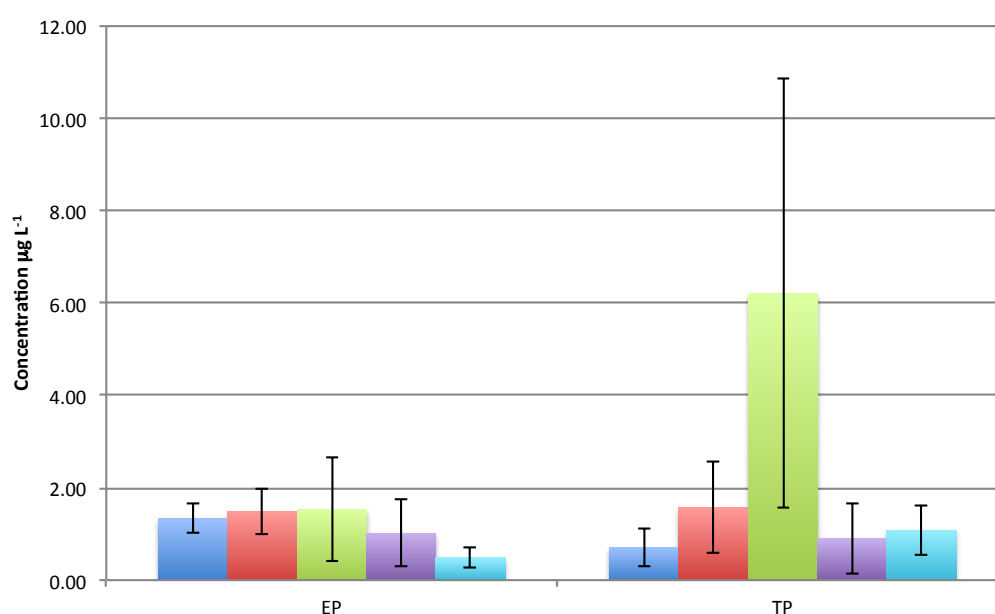


Figure 32 Drug concentration in solution (mean values, $n=3$) obtained for the planted microcosms with enrofloxacin (EP) and tetracycline (TP) after 1; 2; 4; 8; 12 weeks.

The pH results obtained for the planted microcosms along the twelve weeks ranged from 7.3 to 7.7, among the different samples from CP, EP and TP, accordingly to the stability of the system behaviour along time.

Toxicity results for the planted microcosms along the 12 weeks period (Figure 33) reveal effective decrease in wastewater toxicity after treatment along time.

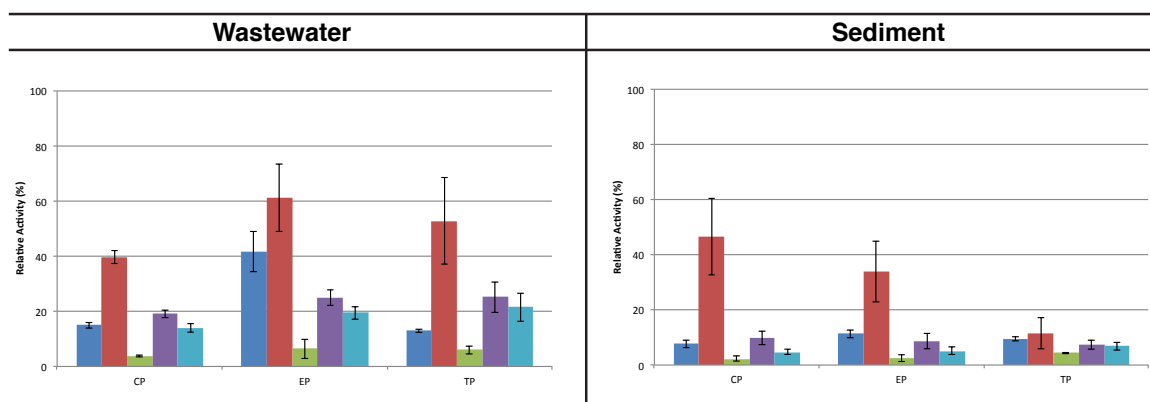


Figure 33 ToxScreen tests measured as relative activity (%) (mean values, n=3) in wastewater and sediment elutriates along the 12 weeks study for the different samples, control planted (CP), enrofloxacin planted (EP), tetracycline planted (TP) after 1; 2; 4; 8; 12 weeks.

Chlorophyll contents (chlorophyll a, b, total chlorophyll and carotenoids content) of the leaves of the plants used in the experiment were determined as a measure of possible induced stress and phytotoxicity of the drugs (Dordio et al. 2009). Previous results for *P. australis* (Chapter 7) pointed to some plant stress due to exposure to the veterinary drugs in accordance with the known fact that ENR may generate both toxic effect and hormesis to plants, related to plant drug uptake (Fatta-Kassinos et al. 2011). In this work, chlorophyll pigments contents presented similar behaviours, being only the total chlorophyll content in plants leaves presented (Figure 34). Although the existence of different behaviours between plants in control and drugs microcosms there was no significant decrease of the chlorophyll content due to the presence of ENR or TET. Present results show that, for the levels of drugs in wastewater $< 100 \mu\text{g L}^{-1}$, occurrence of phytotoxicity in CWs plants will not occur. In CWs planted beds, plants are not directly exposed to the drugs. In addition, it is known that these drugs tend to sorb strongly to soils (Tadeo et al. 2012). So, in the present work, there were no induced stress and phytotoxicity signs indicating that the tested plants were able to cope both with the wastewater (which showed toxicity) and with both drugs tested.

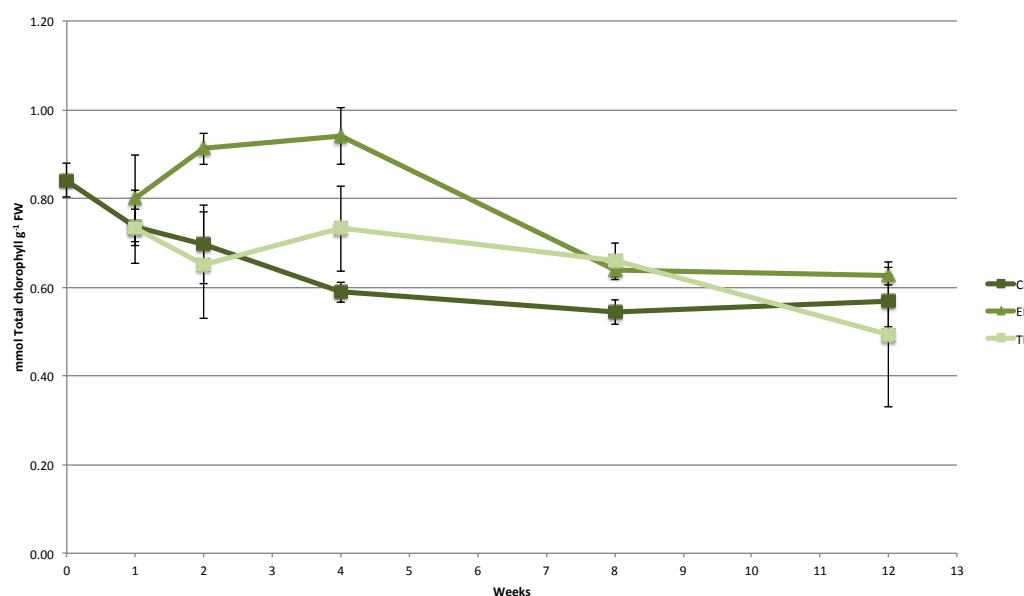


Figure 34 Total chlorophyll content expressed as mmol per gram of leaves fresh weight (FW) of *Phragmites australis* in CWs microcosms: control planted (CP), enrofloxacin planted (EP) and tetracycline planted (TP)

One should be aware that each type of CWs may present different efficiencies. In the present work a vertical subsurface flow CW was simulated. Nevertheless, results obtained are in accordance with those of previous works with CWs applications to other veterinary drugs removal from wastewater, although with CWs of different designs. A constructed macrophyte floating bed system microcosm with three varieties of Italian ryegrass (*Lolium multiflorum* Lam. - Dryan, Waseyutaka and Tachimasari) has provided removal efficiencies between 73 and 99.5 % of sulfonamide antimicrobials (SAs - sulfadiazine, sulfamethazine, and sulfamethoxazole) (Xian et al. 2010). Free water surface CWs revealed removal efficiencies between 26.77 and 34.21% of ionophores (monensin, salinomycin and narasin) (pharmaceuticals used exclusively for veterinary application considered as high-risk compounds) (Hussain et al. 2012).

So, present results are encouraging as they indicate that CWs have potential to mitigate the release of veterinary drugs to the environment. Therefore, the performance of full-scale systems should be assessed.

8.4 Conclusions

Present study indicates that CWs have potential to mitigate the release of veterinary drugs, namely ENR and TET from wastewaters. Removal efficiencies of 94 and 98 % were achieved for TET and ENR, respectively along twelve weeks treating pigfarm treated wastewater with a drug doping level of $100 \mu\text{g L}^{-1}$.

Results showed no occurrence of phytotoxicity in *P. australis* in CWs microcosms. Occurrence of adsorption of the drugs to the substrate may be the predominant mechanism for ENR, although for TET there were signs that degradation was also occurring.

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PART IV

CONCLUSIONS

Chapter 9 - Final conclusions

9.1 Final Conclusions

9.1 Final conclusions

Pharmaceuticals have been detected in the natural environment across the world through a variety of hydrological, climatic, and land-use settings and some can persist in the environment for months to years. Pharmaceuticals in the environment is still an under development thematic at different fields (toxicology; environment monitoring; wastewater treatment) on which this PhD project intended to extend the knowledge.

This research focused on veterinary pharmaceuticals, used extensively as the human ones, but less studied from an environmental point of view. The objectives focused on two complementary areas. The analytical methodology optimization for determination of veterinary drugs on both aqueous and solid matrices was pursued by GC and LC techniques. Investigation of different factors that promote degradation/remediation of those drugs was also performed though application of microcosms studies on activated sludge reactors and CWs.

The set of results obtained in this work suggested that GC is not suitable for the analysis of some veterinary drugs, namely ivermectin, penicillin G and V, ENR and CEF (Chapter 3). Therefore, an expeditious analytical method for simultaneous analysis of five pharmaceuticals of three different families, namely MNC, OTC, TET, ENR and CEF, in aqueous samples through SPE-HPLC-DAD was optimized and characterized (Chapter 4). Developed methodology, despite the fact that LODs are higher than those obtained by other techniques commonly applied (mostly LC-MS methodologies), is an inexpensive and expeditious analytical technique that can be useful for signaling problematic wastewaters effluents.

The SPE-HPLC-DAD methodology was applied to samples of different WWTPs effluents with probability of containing veterinary drugs. This study (Chapter 4) reports for the first time the presence of veterinary drugs in WWTPs effluents in Portugal. The detection of some of the analyzed drugs in some samples shows that these effluents can be an important source of these drugs in the environment and that cost-effective approaches should be found to reduce/eliminate veterinary drugs from wastewater effluents

An expeditious analytical method for simultaneous analysis of the selected five veterinary pharmaceuticals (MNC, OTC, TET, ENR and CEF) in solid samples from WWTPs was also optimized (Chapter 5). For sludge samples the method yielded good selectivity and, taking in account the complexity of the matrix, acceptable recovery results, in accordance with data available in the literature for similar analytical processes. For sediment samples the method yielded lower recovery values, due to the complexity of the sediment matrices, although also in accordance with available data in the literature. These optimized methodologies can be applied to studies of the fate of veterinary drugs in activated sludge processes of WWTPs, constructed wetlands beds and WWTPs recipient water bodies.

Activated sludge reactors microcosms systems provided consistent results with the known fact that the major factor influencing the efficiency of pollutants removal from water is

their ability to interact with solid particles (Chapter 6). Sludge reactors with $100 \mu\text{g L}^{-1}$ initial drug doping charge presented removal rates of 68% for ENR and 76% for TET after a 10-days period. These values represent still considerable concentrations at $\mu\text{g L}^{-1}$ level that remain in the aqueous phase and, consequently, will pass through the WWTP to the receiving environment. Results indicated that sorption to sludge and to the suspended organic matter present in the wastewater were responsible for a significant percentage of drugs removal from wastewater during its biological treatment. This study also emphasizes the fact that measuring only the dissolved fraction of pharmaceuticals in the WWTP effluent may underestimate the loading and risks to the aquatic environment, particularly for highly organic loaded wastewaters such as those of livestock industry. Therefore, veterinary drugs will be a problem for the environment not only due to their presence in the wastewater but also to sludge biosolids reuse and through particulate matter sedimentation. Conventional activated sludge treatment is characterized by shorter hydraulic and solid retention times, indicating that there will be less opportunity for veterinary pharmaceuticals to degrade. The risks associated with effluents containing trace pollutants such as antibiotics remains uncertain, pointing the need for more efficient WWTP technologies.

In order to test the applicability of CWs to the removal of veterinary drugs from wastewaters, initial simplified microcosm tests with the plant *P. australis* were performed (Chapter 7). The plant played an important role in drugs removal from the aquatic medium for a HRT of 7 days. Microbial abundance estimated revealed in general no differences between solutions with and without plants, indicating that microorganisms were not a major participant in drugs removal and/or degradation. Occurrence of adsorption of the drugs to plant roots was observed, however such adsorption was low. Therefore, mechanisms occurring for veterinary drugs removal were plant uptake and/or degradation.

Further tests were performed with complete CWs microcosms (plant and support system) in order to study the potential positive effects of these systems for the reduction/elimination of veterinary pharmaceuticals from livestock and slaughterhouse industries wastewater (Chapter 8). Removal efficiencies of 94 and 98 % were achieved for TET and ENR, respectively, treating pigfarm wastewater effluent doped at a $100 \mu\text{g L}^{-1}$ drug level, along twelve weeks. Results showed no occurrence of phytotoxicity in *P. australis* in CWs microcosms. Occurrence of adsorption of the drugs to the substrate may be the predominant mechanism for ENR removal, although for TET there were signs that degradation was also occurring. Results revealed, therefore, that CWs have potential to mitigate the release of these veterinary drugs, namely a fluoroquinolone and a tetracycline, from wastewaters.

Future perspectives

There are already indications that some pharmaceuticals are indeed problematic as shown by the inclusion of diclofenac in the list of priority substances under the European Water Framework Directive (WFD) or erythromycin in the contaminant candidate list (CCL 3) of the United States Environmental Protection Agency (U.S. EPA). Therefore, it is crucial to face at this stage the mitigation at sources and not let this group of compounds become a historical problem such as persistent organic pollutants (POPs).

Results obtained along the PhD work indicated that CWs have the ability to remove some veterinary antibiotics from wastewater. Therefore, evaluating if full-scale CWs systems have the capability to clean wastewater contaminated with veterinary pharmaceutical compounds, acting as a complement of commonly used biological treatments, deserves further research. In addition, investigation of ways to enhance the processes by which CWs contribute for veterinary drugs removal deserves attention. The type of CWs design may present different efficiencies, e.g. free water surface CWs are prone to potentiate photodegradation of compounds besides the wetland processes. The CWs may be planted with single or mixture of submerged, emerged or floating vegetation. The efficiency of CWs for pollutants removal can be significantly enhanced using support matrixes with capacity to retain contaminants, such as sand, gravel, lava rock, Light Expanded Clay Aggregates (LECA) and cork granulates.

Such topics are crucial on Europe's interests for water supply and consumption covering agricultural water reuse, namely on the Joint Programming Initiatives (JPIs): antimicrobial resistance, water challenges and agriculture, food security and climate change.

APPENDIX

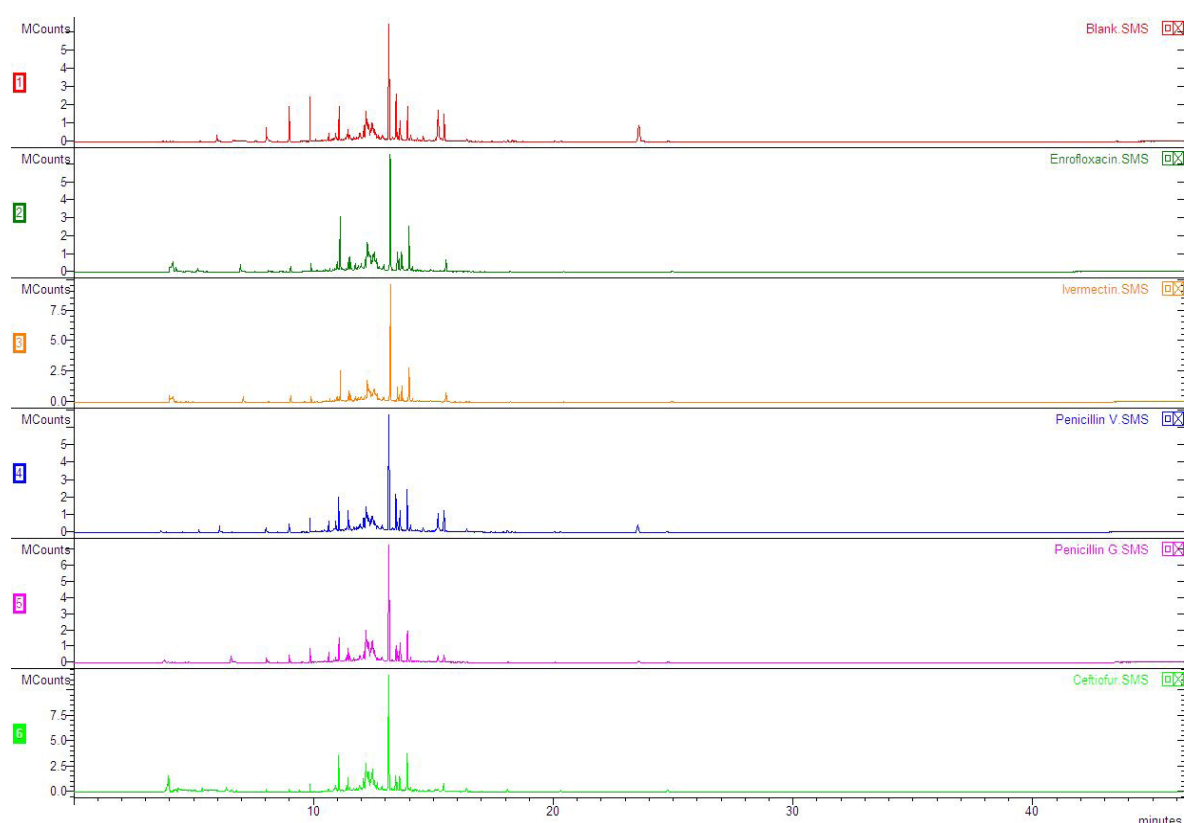


Figure 35 Chromatograms of headspace solid-phase microextraction (SPME) followed by gas chromatography (GC) coupled to mass spectrometry (MS) analysis of aqueous veterinary drugs standards (0.5 mg L^{-1}) derivatized by dimethyl sulphate (DMS).

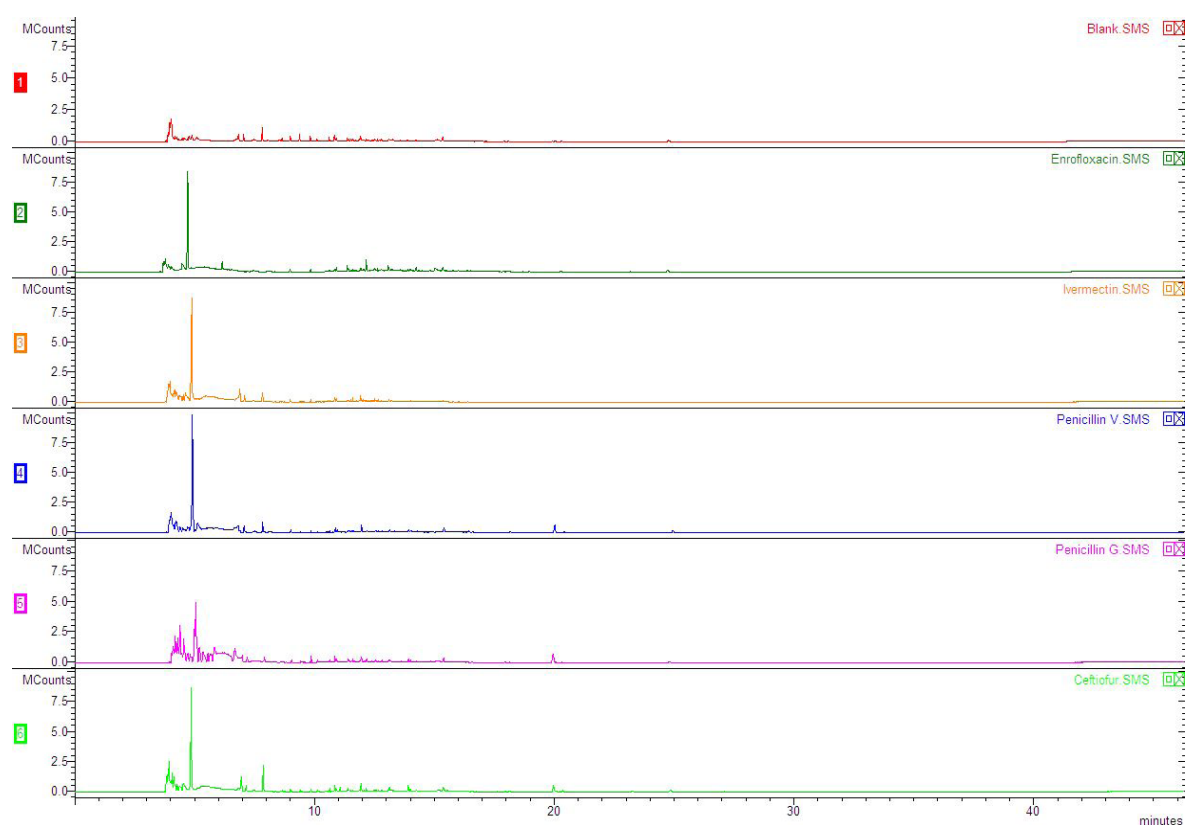


Figure 36 Chromatograms of headspace solid-phase microextraction (SPME) followed by gas chromatography (GC) coupled to mass spectrometry (MS) analysis of aqueous veterinary drugs standards (0.5 mg L⁻¹) derivatized by acetic anhydride (AA).

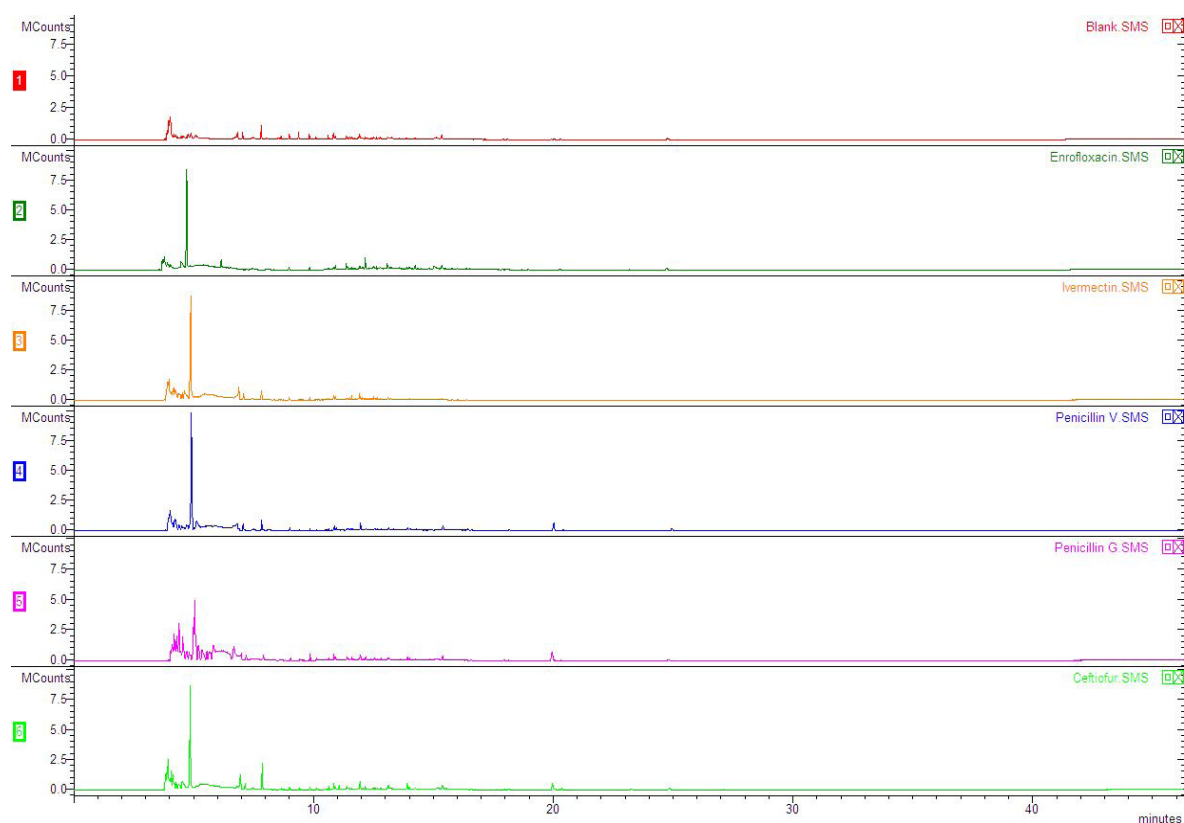


Figure 37 Chromatograms of headspace solid-phase microextraction (SPME) followed by gas chromatography (GC) coupled to mass spectrometry (MS) analysis of aqueous veterinary drugs standards (0.5 mg L^{-1}) derivatized by ethyl chloroformate (ECF).